(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
29 January 2004 (29.01.2004)

PCT

(10) International Publication Number
WO 2004/009126 A1

(51) International Patent Classification[7]: A61K 47/48, C07K 19/00

(21) International Application Number: PCT/GB2003/003082

(22) International Filing Date: 15 July 2003 (15.07.2003)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
0216865.6 19 July 2002 (19.07.2002) GB

(71) Applicant *(for all designated States except US)*: **HEALTH PROTECTION AGENCY** [GB/GB]; Porton Down, Salisbury, Wiltshire SP4 0JG (GB).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **SHONE, Clifford, Charles** [GB/GB]; Health Protection Agency, Porton Down, Salisbury, Wiltshire SP4 0JG (GB). **SUTTON, John, Mark** [GB/GB]; Health Protection Agency, Porton Down, Salisbury, Wiltshire SP4 0JG (GB).

(74) Agents: **SCHLICH, George, William** et al.; Mathys & Squire, 100 Gray's Inn Road, London WC1X 8AL (GB).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 2004/009126 A1

(54) Title: TARGETED AGENTS FOR NERVE REGENERATION

(57) Abstract: A conjugate, for delivery of a therapeutic agent to a neuronal cell, comprises the therapeutic agent, a binding domain that binds to the neuronal cell, and a translocation domain that translocates the therapeutic agent into the neuronal cell, wherein the binding domain is H_c of botulinum C_1 toxin or is based thereon.

TARGETED AGENTS FOR NERVE REGENERATION

The present invention relates to delivery of agents to neuronal cells, especially agents that promote nerve regeneration, to constructs for delivering the agents, to associated use of the agents and constructs and to manufacture thereof.

There are presently few effective treatments for major disorders of the central nervous system. Such disorders include neurodegenerative diseases, stroke, epilepsy, brain tumours, infections and HIV encephalopathy, and sufferers of these diseases far outnumber the morbidity of cancer and heart disease. As our understanding of brain pharmacology increases and the underlying pathologies of diseases are elucidated, potential therapeutic strategies become apparent. All these treatments, however, face the formidable problem of efficient delivery of therapeutics to the various neuronal cell populations involved. Vectors which can effect efficient delivery to neuronal cells are thus required for a broad range of therapeutic substances, including drugs, enzymes, growth factors, therapeutic peptides and genes.

A major problem in the use of such therapies is the delivery of useful concentrations of the active agent to the site of trauma. Specific neuronal vectors could therefore play an important role in targeting such compounds to neuronal cells.

Suitable neuronal cell-specific targeting ligands are therefore required for a broad range of gene vectors to enable effective treatments for neuronal diseases to be developed.

The clostridial neurotoxins are protein toxins produced by various species of the genus *Clostridium*, most importantly *C. tetani* and several strains of *C. botulinum*. There are at present eight different classes of the neurotoxins known: tetanus toxin and botulinum neurotoxin in its serotypes A, B, C_1, D, E, F and G, and they all share similar structures and modes of action. The clostridial neurotoxins are synthesized by the bacterium as a single polypeptide that is modified post-translationally to form two polypeptide chains joined together by a disulphide bond. The two chains are termed the heavy chain (H), and the light chain (LC). The clostridial neurotoxins are

highly selective for neuronal cells, and bind with high affinity thereto.

The botulinum neurotoxins are a sub-family of clostridial neurotoxins whose primary site of action is the neuromuscular junction where they block the release of the transmitter acetylcholine. Tetanus toxin is structurally very similar to botulinum neurotoxins but its primary site of action is the central nervous system where it blocks the release of inhibitory neurotransmitters from central synapses (Renshaw cells).

The neuronal cell targeting of tetanus and botulinum neurotoxins is highly specific and is considered to be a receptor mediated event following which the toxins become internalised and subsequently traffic to the appropriate intracellular compartment where they effect their endopeptidase activity.

It is possible to provide functional definitions of the heavy chain domains within the clostridial neurotoxin molecules, as follows:-

clostridial neurotoxin heavy chain H_C domain:-

- a portion of the heavy chain which is responsible for binding of the native holotoxin to cell surface receptor(s) involved in the intoxicating action of clostridial toxin prior to internalisation of the toxin into the cell.

clostridial neurotoxin heavy chain H_N domain:-

- a portion of the heavy chain which enables translocation of that portion of the neurotoxin molecule such that a functional expression of light chain activity occurs within a target cell.

- the domain responsible for translocation of the endopeptidase activity, following binding of neurotoxin to its specific cell surface receptor via the binding domain, into the target cell.

- the domain responsible for formation of ion-permeable pores in lipid membranes under conditions of low pH.

Tetanus and the botulinum neurotoxins from most of the seven serotypes, together with their derived heavy chains, have been shown to bind a wide variety of neuronal cell types with high affinities in the nM range, e.g. botulinum type B neurotoxin (Evans *et al.* (1986) Eur. J. Biochem. 154, 409-416).

It is known to use H_C domains from botulinum toxins A and B to provide specific targeting of therapeutic agents to neuronal cells.

However, a problem that has been identified with these targeted constructs is that when the H_C domain is made recombinantly, the affinity and specificity for neuronal cells is significantly reduced, and this can be up to the point where there is no effective targeting of neuronal cells. To date, while attempts have been made to modify the H_C regions from A and B toxins to overcome this difficulty there has been no success.

Separately, it is known to use an adenoviral vector to deliver to a neuronal cell a gene that codes for C3 exoenzyme to promote central nervous system axon regeneration. This approach, however, has the disadvantage of the risk of viral proliferation in the patient receiving the treatment. As a result, it would be unlikely ever to receive approval for human use.

An object of the present invention is to provide agents that are targeted to neuronal cells and which can be used to deliver therapeutic agents thereto. An object of specific embodiments of the invention is to provide targeted delivery to neuronal cells of agents that can promote nerve regeneration. A further object is to overcome or at least improve upon the problems and disadvantages identified in the prior art.

The invention provides in its various aspects, targeted delivery of therapeutic agents, e.g. an inhibitor of Rho function, to neural cells, to promote nerve regeneration; and constructs for targeted delivery of therapeutic agents to neuronal cells, using an H_C domain from botulinum C_1 toxin, especially one made recombinantly.

Accordingly, a first aspect of the invention provides a composition, for delivery of a therapeutic agent to a neuronal cell, comprising:-

the therapeutic agent, and

a neuronal cell targeting component, which component comprises a H_C domain of botulinum C_1 toxin, or a fragment, variant, or derivative thereof which retains the function of the native H_C domain.

Thus according to the invention, an H_C portion from botulinum toxin C_1 is made recombinantly and used to provide targeting to neuronal cells of therapeutic agents. The H_C portion retains its binding affinity for neuronal cells, in contrast to the corresponding chains from A and B toxins.

Compositions of the invention suitably further comprise a translation domain, for translocation of the therapeutic agent into a target cell. The neuronal cell targeting agent may be linked, e.g. covalently, using linkages which may include one or more spacer regions, to a translocation domain to effect transport of the therapeutic agent into the cytosol. Examples of translocation domains derived from clostridial neurotoxins are as follows:-

Botulinum type A neurotoxin	– amino acid residues (449-871)
Botulinum type B neurotoxin	– amino acid residues (441-858)
Botulinum type C neurotoxin	– amino acid residues (442-866)
Botulinum type D neurotoxin	– amino acid residues (446-862)
Botulinum type E neurotoxin	– amino acid residues (423-845)
Botulinum type F neurotoxin	– amino acid residues (440-864)
Botulinum type G neurotoxin	– amino acid residues (442-863)
Tetanus neurotoxin	– amino acid residues (458-879)

Other clostridial sources of translocation domains include - *C. butylicum*, and *C. argentinense,* and for the genetic basis of toxin production in *Clostridium botulinum* and *C. tetani*, see Henderson *et al* (1997) in *The Clostridia: Molecular Biology and Pathogenesis, Academic press.*

In addition to the above translocation domains derived from clostridial sources, other non-clostridial sources may be employed in a construct according to the present invention. These include, for example, diphtheria toxin [London, E. (1992) *Biochem. Biophys. Acta., 1112, pp.25-51*], Pseudomonas exotoxin A [Prior *et al* (1992) *Biochem., 31, pp.3555-3559*], influenza virus haemagglutinin

fusogenic peptides [Wagner *et al* (1992) *PNAS, 89, pp.7934-7938*], and amphiphilic peptides [Murata *et al* (1992) *Biochem., 31, pp.1986-1992*].

In preferred embodiments of the invention, a translocation domain is selected from botulinum toxin C_1 translocation domain and functional fragments and derivatives thereof, and diphtheria toxin translocation domain and functional fragments and derivatives thereof.

In use, the domains of a construct according to the present invention are associated with each other. In one embodiment, two or more of the domains may be joined together either directly (eg. by a covalent linkage), or via a linker molecule. Conjugation techniques suitable for use in the present invention have been well documented:-

Chemistry of protein conjugation and cross-linking. Edited by Wong, S. S. 1993, CRC Press Inc., Florida; and

Bioconjugate techniques, Edited by Hermanson, G. T. 1996, Academic Press, London, UK.

Direct linkage of two or more domains is now described with reference to embodiments employing clostridial neurotoxins or fragments thereof and to the following nomenclature of clostridial neurotoxin domains, namely Domain B (contains the neuronal cell targeting domain), Domain T (contains the translocation domain) and Domain E (contains the therapeutic agent), although no limitation thereto is intended.

In one embodiment of the present invention, Domains E and T may be mixed together in equimolar quantities under reducing conditions and covalently coupled by repeated dialysis (eg. at 4° C, with agitation), into physiological salt solution in the absence of reducing agents. At this stage, in contrast to Example 6 of WO94/21300, the E-T complex is not blocked by iodoacetamide, therefore any remaining free –SH groups are retained. Domain B is then modified, for example, by derivatisation with the coupling agent SPDP followed by subsequent reduction. In this reaction, SPDP does not remain attached as a spacer molecule to Domain B, but simply increases the efficiency of this reduction reaction. Reduced Domain B and the E-T complex may then be

mixed under non-reducing conditions (eg. at 4 °C) to form a disulphide-linked E-T-B construct.

In another embodiment, a coupled E-T complex may be prepared according to Example 6 of WO94/21300, including the addition of iodoacetamide to block free sulphydryl groups. However, the E-T complex is not further derivatised, and the remaining chemistry makes use of the free amino ($-NH_2$) groups on amino acid side chains (eg. lysine, and arginine amino acids).

Domain B may be derivatised using carbodiimide chemistry (eg. using EDAC) to activate carboxyl groups on amino acid side chains (eg. glutamate, and aspartate amino acids), and the E-T complex mixed with the derivatised Domain B to result in a covalently coupled (amide bond) E-T-B construct.

Suitable methodology for the creation of such a construct is, for example, as follows:-

Domain B was dialysed into MES buffer (0.1 M MES, 0.1 M sodium chloride, pH 5.0) to a final concentration of 0.5mg/ml. EDAC (1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride) was added to final concentrations of 0.2 mg/ml and reacted for 30 min at room temperature. Excess EDAC was removed by desalting over a MES buffer equilibrated PD-10 column (Pharmacia). The derivatised domain B was concentrated (to >2mg/ml) using Millipore Biomax 10 concentrators. The E-T complex (1 mg/ml) was mixed for 16 hours at 4 °C, and the E-T-B complex purified by size-exclusion chromatography over a Superose 12 HR10/30 column (Pharmacia) to remove unreacted Domain B (column buffer: 50mM sodium phosphate pH6.5 + 20mM NaCl).

As an alternative to direct covalent linkage of the various Domains of a construct / composition according to the present invention, suitable linker molecules may be employed. The term linker molecule is used synonymously with spacer molecule. Spacer technology was readily available prior to the present application.

For example, one particular coupling agent (SPDP) is described in Example 6 of WO94/21300 (see lines 3-5 on page 16). In Example 6, SPDP is linked to

an E-T complex, thereby providing an E-T complex including a linker molecule. This complex is then reacted with a Domain B, which becomes attached to the E-T complex via the linker molecule. In this method, SPDP results in a spacing region of approximately 6.8 Angstroms between different Domains of the construct of the present invention.

A variant of SPDP known as LC-SPDP is identical in all respects to SPDP but for an increased chain length. LC-SPDP may be used to covalently link two Domains of the construct of the present invention resulting in a 15.6 Angstrom spacing between these Domains.

Examples of spacer molecules include, but are not limited to:-

$(GGGGS)_2$, elbow regions of Fab	- [see Anand *et al.* (1991) J. Biol. Chem. **266**, 21874-9];
$(GGGGS)_3$	- [see Brinkmann *et al.* (1991) Proc. Natl. Acad. Sci. **88**, 8616-20];
the interdomain linker of cellulase	- [see Takkinen *et al.* (1991) Protein Eng, **4**, 837-841];
PPPIEGR	- [see Kim (1993) Protein Science, 2, 348-356];
Collagen-like spacer	-[see Rock (1992) Protein Engineering, vol 5, No 6, pp583-591]; and
Trypsin-sensitive diphtheria toxin peptide	- [see O'Hare (1990) FEBS, vol 273, No 1,2, pp 200-204].

In a further embodiment of the present invention, a construct having the structure E-X-T-X-B, where "X" is a spacer molecule between each domain, may be prepared, for example, as follows:-

Domain E is derivatised with SPDP, but not subsequently reduced. This results

in an SPDP-derivatised Domain E. Domain T is similarly prepared, but subsequently reduced with 10mM dithiothreitol (DTT). The 10mM DTT present in the Domain T preparation, following elution from the QAE column (see Example 6 in WO94/21300), is removed by passage of Domain T through a sephadex G-25 column equilibrated in PBS. Domain T free of reducing agent is then mixed with the SPDP-derivatised Domain E, with agitation at 4 °C for 16 hours. E-T complex is isolated from free Domain E and from free Domain T by size-exclusion chromatography (Sephadex G-150). Whereafter, the same procedure can be followed as described in Example 6 of WO94/21300 for rederivatisation of the E-T complex with SPDP, and subsequent coupling thereof to the free sulphydryl on Domain B to form.

In a construct of the invention, the translocation domain (Domain T) is not limited, and could be any translocation domain. Translocation domains can frequently be identified by the property of being able to form measurable pores in lipid membranes at low pH (Shone *et al.* Eur J. Biochem. 167, 175-180).

The therapeutic agent could, similarly, be any suitable agent and reference to "therapeutic substance" or "therapeutic agent" is a reference to any substance, agent or mixture thereof, which, if delivered by or in the composition of the invention, is beneficial in the treatment of disease. Examples of these include drugs, growth factors, enzymes, and DNA packaged in various forms (e.g. modified viruses, cationic liposomes, and condensed DNA).

For promoting nerve regeneration, any Rho inhibitor can be used, especially a C3 exoenzyme. The C3 family of exoenzymes is defined as a family of proteins of 20-30kDa with a basic isolectric point usually greater than 9. The enzymes are ADP-ribosyltransferases, which modify small GTPases, usually of the Rho family, by the addition of ADP-ribose. This usually results in the inactivation of the GTPases.

Members of the C3 exoenzyme family described to date include at least 2 isoforms from *Clostridium botulinum*, an isoform from *C.limosum*, an isoform from *Bacillus cereus*, and 3 isoforms from *Staphylococcus aureus*. C3 isoforms from *C.acetobutylicum*, *Streptococcus pyogenes* and *Listeria monocytogenes* are disclosed for the first time herein and represent further embodiments of the invention.

Enzymes having C3 activity can be identified, for example, using the assay described in Example 1.

C3-like toxins can be further defined by the presence of motifs covering two elements of the active site such that all examples carry the consensus S S/T S/T Hyd X35-40 Q/E X E Hyd Hyd Hyd where Hyd represents any hydrophobic residue (usually I, L, V, G or A) and X represents any amino acid. The motif usually has an aromatic residue in one of the two preceding positions. The S S/T S/T motif is a key determinant of substrate specificity. Hence, a C3 enzyme used in the present invention is preferably one that includes these motifs.

Further aspects of the invention provide a polypeptide construct comprising an inhibitor of a member of the Rho family of GTPases, for use in neuronal cell therapy; and use of an inhibitor of a member of the Rho family of GTPases, for use in manufacture of a medicament for neuronal cell therapy, the inhibitor in both cases suitably being or comprising a C3 enzyme.

In use, advantages of constructs of the invention include one or more of:-

targeted to the neuron, therefore no cytotoxic effect on non-neuronal cells,

the enzymic action of C3, in which one C3 molecule can inactivate many target (Rho) molecules, provides efficient inhibition which is more effective than conventional (drug-like) inhibitors which act by binding to their target in a one-to-one ratio,

much smaller molecular size compared to virus vectors, therefore better penetration of tissues *in vivo*,

better safety profile compared to viral delivery vectors with no risk of uncontrolled replication *in vivo,*

delivery of functional protein to cells with no delay in action which contrasts to DNA delivery in which translation of the DNA into the protein has to occur before an effect,

constructs of the invention are less immunogenic than viral or DNA constructs,

constructs prepared using C3 from *Staphylococcus* species have the advantage that they are not inhibited by the naturally occurring intracellular protein RalA,

botulinum toxin-targeted C3 rapidly bind to neurons due to their high affinity receptor interaction resulting in efficient action and reduced immune responses, and

possible to specifically target Rho isoforms other than the cytoplasmic RhoA; for example, targeting of the endosomal RhoB has significant therapeutic potential.

A modified clostridial heavy chain for the composition of the invention is suitably produced by combining the binding domain (H_C domain) of a clostridial neurotoxin with a non-clostridial translocation domain. Thus, for example, a modified clostridial heavy chain fragment may be constructed from the translocation domain of diphtheria toxin (residues 194-386) fused to the H_C domain of a botulinum toxin (e.g. type F H_C fragment, residues 865-1278; type A H_C fragment, residues 872-1296).

In another embodiment of the invention, the modified clostridial heavy chain is produced by combining the H_C domain of a clostridial neurotoxin with a membrane disrupting peptide which functions as a translocation domain, suitably a viral peptide. Thus, for example, a modified clostridial heavy chain fragment may be constructed by combining the H_C domain of a botulinum toxin with a peptide based on influenza virus haemagglutinin HA2 (residues 1-23).

In another embodiment of the invention, the modified clostridial heavy chain fragment is fused to a linker peptide via the N-terminus of the translocation domain, to which linker peptide a polypeptide payload may be attached. An example of such a linker peptide is the sequence CGLVPAGSGP (SEQ ID NO:23) which contains the thrombin protease cleavage site and a cysteine residue for disulphide bridge formation. Such a peptide linker allows

production of a recombinant fusion protein comprising a polypeptide therapeutic molecule fused by the linker peptide to the N-terminus of the modified clostridial heavy chain fragment. The latter single chain fusion protein may then be treated with thrombin to give a dichain protein in which the polypeptide therapeutic is linked to the translocation domain of the modified clostridial heavy chain fragment by a disulphide link between a free cysteine on the translocation domain and a cysteine residue of the linker peptide. In another example of a linker peptide in which the translocation domain does not contain a free cysteine residue near its C-terminus, such as is the case when the translocation domain is a fusogenic peptide, the linker peptide contains both cysteine residues required for the disulphide bridge. An example of the latter linker peptide is the amino acid sequence: CGLVPAGSGPSAGSSAC (SEQ ID NO:24).

In another embodiment of the invention, the modified clostridial heavy chain is linked to a polypeptide which may be an enzyme, growth factor, protein or peptide which has therapeutic benefits when delivered to neuronal cells. The polypeptide may be linked to the modified clostridial heavy chain by chemical means. Alternatively, the polypeptide may be produced as a fusion protein linked to the modified clostridial binding fragment by recombinant technology using the linker peptides as described above. In such an example, the construct would contain the following components:-

a polypeptide therapeutic substance;
a linker peptide; and
a modified clostridial heavy chain

In yet another embodiment of the invention, the modified clostridial heavy chain is linked directly or indirectly to DNA such that the construct is capable of delivering the DNA to neuronal cells. Such constructs have gene therapy applications and be used to switch on, or off, selected genes with the cell. The DNA may be contained within a liposome or be condensed via a peptide or protein. The modified clostridial heavy chain may be chemically linked to the protein that effects the DNA condensation by chemical coupling agents. Alternatively, the modified clostridial heavy chain may be produced as a fusion protein, by recombinant technology, with a peptide that can effect the condensation of DNA.

In yet another embodiment of the invention, the modified clostridial heavy chain fragment may be linked to a recombinant virus such that the modified virus has an altered tropism and is capable of transducing cells. Such a construct is of use to correct genetic defects within neuronal cells by switching on, or off, selected genes. The modified clostridial heavy chain fragment may be linked directly to the surface of the virus using chemical cross-linking agents. Alternatively the modified clostridial heavy chain fragment may be linked to the recombinant virus via an antibody which specifically bind to the virus. In this instance the modified clostridial heavy chain fragment is chemically coupled to a polyclonal or monoclonal antibody which specifically recognizes a marker on the surface of the virus. A similar modified clostridial heavy chain fragment-antibody fusion protein could be produced by recombinant technology in which the antibody component is a recombinant single chain antibody.

In yet another embodiment of the invention, the modified clostridial heavy chain fragment is linked to a drug release system such as a microparticle constructed from a suitable polymer, e.g. poly (lactide-co-glycolide), polyhydroxylalkonate, collagen, poly(divinyl-ether-comaleic anhydride, poly (styrene-co-maleic anhydride) or other polymer useful in such microparticles. The modified clostridial heavy chain fragment may be linked to the drug release system by covalent chemical coupling, or electrostatic or hydrophobic forces. The modified clostridial heavy chain fragment may also be encapsulated within the release vehicle together with the therapeutic payload provided that a portion of the modified clostridial binding domain is exposed at the surface. Alternatively, the modified clostridial heavy chain fragment may be linked, at either the N– or C-terminal end, to a peptide or protein to facilitate coupling of the fragment to the drug release system.

Other strategies are known by which modified clostridial heavy chain fragments can be linked to range of therapeutic substances using a variety of established chemical cross-linking techniques, and a variety of fusion proteins can be produced containing a modified clostridial binding fragment and another polypeptide. Using these techniques a variety of substances can be targeted to neuronal cells using the modified clostridial heavy chain fragments. Examples of possible uses of the modified clostridial heavy chain fragments as neuronal delivery vectors are given in more detail below in

Constructs of the invention may be introduced into either neuronal or non-neuronal tissue using methods known in the art. By subsequent specific binding to neuronal cell tissue, the targeted construct exerts its therapeutic effects. Ideally, the construct is injected near a site requiring therapeutic intervention.

The construct of the invention may be produced as a suspension, emulsion, solution or as a freeze dried powder depending on the application and properties of the therapeutic substance. The construct of the invention may be resuspended or diluted in a variety of pharmaceutically acceptable liquids depending on the application.

"Clostridial neurotoxin" means either tetanus neurotoxin or one of the seven botulinum neurotoxins, the latter being designated as serotypes A, B C_1, D, E, F or G.

"Modified clostridial heavy chain fragment" means a polypeptide fragment which binds to neuronal cell receptors in similar manner to a corresponding heavy chain derived from botulinum or tetanus toxins but differs in its amino acid sequence and properties compared to the corresponding fragment derived from botulinum or tetanus toxin.

"Bind" in relation to the botulinum and tetanus heavy chain fragments, means the specific interaction between the clostridial fragment and one or more cell surface receptors or markers which results in localization of the binding fragment on the cell surface. In the case of the clostridial neurotoxins, the property of a fragment being able to 'bind' like a fragment of a given serotype can be demonstrated by competition between the ligand and the native toxin for its neuronal cell receptor.

"High affinity binding specific to neuronal cell corresponding to that of a clostridial neurotoxin" refers to the ability of a ligand to bind strongly to cell surface receptors of neuronal cells that are involved in specific binding of a given neurotoxin. The capacity of a given ligand to bind strongly to these cell surface receptors may be assessed using conventional competitive binding assays. In such assays radiolabelled clostridial neurotoxin is contacted with neuronal cells in the presence of various concentrations of non-radiolabelled

ligands. The ligand mixture is incubated with the cells, at low temperature (0-3°C) to prevent ligand internalization, during which competition between the radiolabelled clostridial neurotoxin and non-labelled ligand may occur. In such assays when the unlabelled ligand used is the same as that of the labelled neurotoxin, the radiolabelled clostridial neurotoxin will be displaced from the neuronal cell receptors as the concentration of non-labelled neurotoxin is increased. The competition curve obtained in this case will therefore be representative of the behaviour of a ligand which shows "high affinity binding specificity to neuronal cells corresponding to that of a clostridial neurotoxin", as used herein.

"Translocation domain" means a domain or fragment of a protein which effects transport of itself and/or other proteins and substances across a membrane or lipid bilayer. The latter membrane may be that of an endosome where translocation will occur during the process of receptor-mediated endocytosis. Translocation domains can frequently be identified by the property of being able to form measurable pores in lipid membranes at low pH (Shone *et al.* Eur J. Biochem. 167, 175-180). Examples of translocation domains are set out in more detail below in Figure 1. In the application, translocation domains are frequently referred to as "H_N domains".

"Translocation" in relation to translocation domain, means the internalization events which occur after binding to the cell surface. These events lead to the transport of substances into the cytosol of neuronal cells.

"Therapeutic substances" or "agents" mean any substance, agent or mixture thereof, which, if delivered by the modified clostridial binding fragment, would be beneficial to the treatment of neuronal diseases. Examples of these include drugs, growth factors, enzymes, and DNA packaged in various forms (e.g. modified viruses, cationic liposomes, and condensed DNA).

"C3-like activity" means that an enzyme expresses an ADP-ribosyl transferase activity which is specific for small cellular GTPases of the Rho family. In this activity, the C3-like enzyme catalyses the transfer of an ADP-ribose moiety from NAD to the Rho GTPase which usually results in an loss of function of the GTPase.

Also provided in the present invention are methods of manufacture of the polypeptides of the invention by expressing in a host cell a nucleic acid encoding the polypeptide, and the use of a polypeptide or a composition according to the invention in the treatment of a disease state associated with neuronal cells.

The invention is now illustrated in the following specific embodiments and accompanied by drawings in which:-

Fig. 1 shows protection against neurite reaction in LPA-treated neuroblastoma cells by C3 enzyme; and

Fig. 2 shows protective or neurostimulatory effect of C3 on LPA-treated neurons.

Examples:

1. Measurement of exoenzyme C3 activity within an enzyme

To carry out an assay for the exoenzyme C3, an assay mix was prepared containing the following components:-

- 10µl of Assay Buffer (0.05M Hepes pH 7.2 containing $MgCl_2$ to a final concentration of 2mM)
- 20µl of recombinant Rho A solution in Assay Buffer (to give 0.5µg per 100µl assay)
- 20µl of [^{32}P] NAD^+ solution in Assay Buffer (to give 1µCi per 100µl assay). The NAD being labelled in the alpha phosphate position.

This mixture was incubated at 37°C for 5 min then 50µl of C3 enzyme solution (prewarmed to 37°C) added to start the enzymic reaction.

After incubation for various times at 37°C (typically between 15-120min), 100µl of BSA (2mg/ml) was added to each assay mix followed by 0.5ml of 24% trichloroacetic acid solution (TCA) to stop the reaction. The resulting precipitate was centrifuged at high speed on a microfuge for 2 mins, the

supernatant fluid decanted and then the precipitate washed with a further 1 ml of 12%TCA solution. The precipitate was resuspended in 1ml of PBS, by passaging several times through a pipette tip, and then 5ml scintillation fluid added, mixed and counted in scintillation counter.

The presence of exoenzyme C3, or an enzyme with exoenzyme C3-like activity, was confirmed by the presence of significantly higher radioactivity in the pellet compared to control incubations containing no C3 enzyme.

2. Cloning and expression of C3 genes.

Standard molecular biology protocols were used for all genetic manipulations (Sambrook *et al* 1989, Molecular cloning; A laboratory manual. Second Edition, Cold Spring Harbor Laboratory Press, New York.). C3 genes were amplified by PCR to generate suitable restriction sites for cloning. In some cases synthetic genes were prepared with codon usage optimised for expression in *E.coli*. The restriction sites *Bam*HI (5') and *Bgl*II (3') were used for most cloning operations with reading frames designed to start with the first base of the restriction site. Constructs were sequenced to confirm the presence of the correct sequence. An expression vector containing the malE gene from the pMAL-C2x (NEB) subcloned as an *Apa*I-*Hind*III fragment into the cloning vector pBC (Strategene) digested *Apa*I-*Hind*III was prepared. The C3 genes were cloned into the *Bam*HI site as *Bam*HI-*Bgl*II fragments. Alternatively the C3 genes were cloned into either an expression vector carrying a T7 polymerase promoter site (e.g. pET28, pET30 or derivatives (Novagen Inc, Madison, WI)) or as a fusion with maltose binding protein (e.g. pMALc2x (NEB)) as a suitable fragment. Clones with confirmed sequences were used to transform expression hosts. Strain TB1 was used for most expression with alternative hosts used as required (For T7 polymerase vectors *E.coli* BL21 (DE3) (Studier and Moffatt 1986 *Journal of Molecular Biology* 189:113-130) JM109 (DE3) or equivalent strains with a DE3 lysogen. For pMalc2x JM109, BL21, TG1, TB1 or other suitable expression strains).

In addition to the expression of C3 proteins as standard fusion proteins an additional approach was used to generate fusions for direct assembly into targeting vectors. C3 fragments were cloned into a BamHI site introduced at the 5' end of a neuronal cell targeting moiety such as a hybrid diphtheria

translocation domain-clostridial neurotoxin binding domain fusion protein

Expression cultures of TB1 pBCmalE C3 were grown in Terrific Broth containing 35µg/ml chloramphenicol and 0.5% (w/v) glucose to an OD_{600} of 2.0 at 30°C and cultures were induced with 500µM IPTG and grown at 25°C for 2 hours. Other expression systems used similar conditions except that the antibiotic was changed to either kanamycin or ampicillin. Cells were lysed by either sonication in column buffer (20mM Hepes 100mM NaCl pH 6.8) or suitable detergent treatment (e.g. Bugbuster reagent; Novagen) and cell debris pelleted by centrifugation. Supernatant proteins were loaded onto a SP-sepharose Fast Flow column equilibrated in column buffer and proteins eluted with a gradient of 0-1M NaCl. MBP-C3 fusions eluted at 200mM NaCl. Fusion protein was cleaved with Factor Xa in 20mM Hepes 200mM NaCl pH6.8 (as eluted) to separate the C3 domain from its MBP fusion partner. The protein was diluted to give a final buffer concentration of 20mM Hepes 100mM NaCl pH 6.8 and loaded onto the SP-Sepharose column under identical conditions to those used initially. C3 protein,essentially free of contaminating proteins, was eluted at ~400 mM NaCl. Protein was stored at –70°C until required.

The C3stau2 isoform can be purified using a similar method. Cells are lysed in 20mM MES 50mM NaCl pH5.8 and the soluble material loaded on to an SP-sepharose column. The protein elutes at around 150mM NaCl. The protein is dialysed against 20mM HEPES 50mM NaCl pH 7.3 and cleaved with Factor Xa. The protein is passed through a second SP-sepharose column and elutes at approximately 250mM NaCl. Protein is stable at –70°C.

His-tag fusions were loaded onto a metal chelate column charged with Cu^{2+} (Amersham-Pharmacia Biotech, Uppsala, Sweden). After loading proteins on the column and washing, proteins were eluted using imidazole. All buffers were used as specified by manufacturers. Where appropriate removal of the purification tag was carried out according to manufacturers instructions.

MBP fusions could also be purified on amylose resin columns as described by the manufacturer (NEB) following growth in Terrific Broth containing 100 µg/ml ampicillin and lysis as described above.

3. Expression of heavy chain fragments.

Standard molecular biology protocols were used for all genetic manipulations (Sambrook *et al* 1989, Molecular cloning; A laboratory manual. Second Edition, Cold Spring Harbor Laboratory Press, New York.) Clostridial neurotoxin binding domains (BoNT/Hc or TeNT/Hc) derived from either their native genes or synthetic genes with codon usage optimised for expression in *E.coli* were amplified by PCR. Introduced *Bam*HI (5') restriction sites and *Hind*III, *Sal*I or *Eco*RI (3') sites were used for most cloning operations with reading frames designed to start with the first base of the restriction site. Constructs were sequenced to confirm the presence of the correct sequence. The translocation domain of diphtheria toxin (DipT) was amplified from its native gene to introduce *Bam*HI and *Bgl*II sites at the 5' and 3' ends respectively. This *Bam*HI and *Bgl*II fragment was subcloned into the *Bam*HI site at the 5' end of the Hc fragment to generate an in-frame fusion. The entire heavy chain fragment (DipT-Hc) was excised as a *Bam*HI-*Hind*III or *Bam*HI-*Sal*I or *Bam*HI-*Eco*RI fragment and subcloned into a suitable expression vector.

Constructs for expression were subcloned into either an expression vector carrying a T7 polymerase promoter site (e.g. pET28, pET30 or derivatives (Novagen Inc, Madison, WI)) or to generate a fusion with maltose binding protein (e.g. pMALc2x (NEB)) as a suitable fragment. Clones with confirmed sequences were used to transform expression hosts: For T7 polymerase vectors *E.coli* BL21 (DE3) (Studier and Moffatt 1986 *Journal of Molecular Biology* 189:113-130) JM109 (DE3) or equivalent strains with a DE3 lysogen. For pMalc2x JM109, BL21, TG1, TB1 or other suitable expression strains.

The recombinant proteins expressed from pET vectors contain amino-terminal histidine (6-His) and T7 peptide tags allowing proteins to be purified by affinity chromatography on either a Cu^{2+} charged metal chelate column. Expression cultures were grown in Terrific Broth containing 30µg/ml kanamycin and 0.5% (w/v) glucose to an OD_{600} of 2.0 and protein expression was induced with 500µM IPTG for 2 hours. Cells were lysed by either sonication or suitable detergent treatment (e.g. Bugbuster reagent; Novagen), cell debris pelleted by centrifugation and the supernatant loaded onto a metal chelate column charged with Cu^{2+} (Amersham-Pharmacia Biotech, Uppsala, Sweden). After

loading proteins on the column and washing, proteins were eluted using imidazole. All buffers were used as specified by manufacturers. Where appropriate removal of the purification tag was carried out according to manufacturers instructions.

MBP fusions were purified on amylose resin columns as described by the manufacturer (NEB) following growth in Terrific Broth containing 100 µg/ml ampicillin and lysis as described above.

4. Purification of the heavy chain of botulinum Type C_1 neurotoxin.

Step 1. Botulinum type C_1 neurotoxin (5mg total) is dialysed against borate/phosphate buffer pH 8.5 (29 mM $Na_2B_4O_7$; 42 mM NaH_2PO_4 titrated to pH 8.5 with NaOH) and then applied (0.5ml min^{-1}) to a column (1cm x 5cm) of QAE-Sephadex (Pharmacia) equilibrated in the borate/phosphate pH 8.5 buffer. The column is then washed with a further 10ml of buffer followed by 10ml of the borate/phosphate buffer containing 10mM dithiothreitol. After the latter buffer has been allowed to run almost completely onto the column, 3ml of borate/phosphate pH 8.5 buffer containing 100mM dithiothreitol and 2M urea are carefully applied to the column and 2.5 ml of this buffer allowed to run onto the gel. The column is then sealed and incubated at 4°C overnight.

Step 2. The light subunit of the neurotoxin is eluted with 20ml of borate/phosphate pH 8.5 buffer containing 2M urea and 10mM dithiothreitol collecting 1.5ml fractions.

Step 3. The heavy subunit of the type C_1 neurotoxin is eluted with 20ml of borate/phosphate pH 8.5 buffer containing 2M urea, 10mM dithiothreitol and 0.2M NaCl. The presence of heavy chain in eluted fractions (1ml) may be detected by uv measurement. Those fractions containing the highest concentration of protein are pooled and then dialysed against 0.05M Hepes pH 7.4 buffer containing 0.15M NaCl. The purified type C_1 neurotoxin may be stored frozen at –80°C until required.

5. Preparation of chemical C3-heavy chain conjugates.

Purified C3 was dialysed into phosphate buffered saline (PBS) pH7.4. Sulfo-

LC-SPDP was added to give a final molar excess of 3:1 SPDP:C3 and reacted for 2 hours at room temperature. Free SPDP was removed by dialysis. Derivitised C3 was incubated with either native or recombinant heavy chain fragments containing a free cysteine residue for 20 hours at 4^0C. Free C3 could be removed if required by cation exchange chromatography on a MonoS column run under the same conditions as the SP-Sepharose column described in example 1. Additional purification is also possible on a Cibacron-Blue affinity resin if required.

6 Assessment of activity of C3 conjugates on neuroblastoma cells.

NG108 neuroblastoma cells were cultured in DMEM containing 10% foetal calf serum (FCS) and 2 mM glutamine at 37^0C at 5% CO_2 saturation. Cells were plated onto poly-D-lysine coated 96 well culture plates at a density of $2x10^4$. After 24 hours the cells were changed into DMEM/glutamine medium lacking FCS and grown for a further 16 hours as above. Conjugate or free C3 was added and the cells incubated for either 3 hours or overnight. The cells were treated with 1µM lyso-phosphatidic acid (LPA) in DMEM/glutamine. Cells were observed for morphological changes under a microscope. As shown in Figure 1 C3 at a final concentration of 30µg/ml protected the cells from LPA mediated neurite retraction. To assay for cellular events in response to LPA treatment an MTT assay was used as described previously (Alley MC, Scudiero DA, Monks A, Hursey ML, Czerwinski MJ *et al* (1988) Feasibility of drug screening with panels of human tumor cell lines using a microculture tetrazolium assay. *Cancer Res* 48, 589-601). Assay results shown in Figure 2 show a marked reduction in mitochondrial activity, as measured by reduction of MTT, in LPA treated cells. Cells pre-treated with C3 (30µg/ml final concentration) before addition of LPA at showed similar levels of mitochondrial activity to untreated control cells indicating that C3 prevents LPA-induced cellular damage. This is indicative of protective or neurostimulatory effect in damaged neurons.

Expressed Sequence identifications.

In the sequence listing accompanying this specification, the sequences have the following derivation:-

Sequence 1 C3 protein from *Clostridium botulinum* lacking the N-terminal signal sequence and including a factor Xa cleavge site immediately adjacent to the initial Alanine amino acid of the mature sequence.

Sequence 2 C3 protein from *Staphylococcus aureus* lacking the N-terminal signal sequence and including a factor Xa cleavage site immediately adjacent to the initial Alanine amino acid of the mature sequence. The protein refers to the C3Stau 2 isoform sometimes referred to as EDIN B

Sequence 3 Gene encoding C3 protein from *Staphylococcus aureus* lacking the N-terminal signal sequence and including a factor Xa cleavage site immediately adjacent to the initial Alanine amino acid of the mature sequence. The synthetic gene, with codon usage optimised for expression in *E.coli*, encodes the C3Stau 2 isoform sometimes referred to as EDIN B

Sequence 4 mature C3 protein from *Staphylococcus aureus*; termed C3Stau 1 (EDIN A)

Sequence 5 full length C3 protein from *Staphylococcus aureus*; termed C3Stau 1 (EDIN A) including a signal sequence at the N-terminus.

Sequence 6 mature C3 protein from *Clostridium limosum*

Sequence 7 C3-like protein from *Listeria monocytogenes*

Sequence 8 C3-like protein from *Clostridium acetobutylicum*

Sequence 9 C3-like protein from *Streptococcus pyogenes* (native protein sequence)

Sequence 10 Second C3-like protein from *Streptococcus pyogenes* (native protein sequence)

Sequence 11 shows the native heavy chain (HC) of BoNT/C1 with the di-chain linker region. The native Factor Xa cleavage site within the linker region is also present. Optionally the fragment can also include the amino acids GS at the N-terminus, encoded by an in-frame *Bam*HI site used for

cloning purposes.

Sequence 12 shows the isolated receptor binding domain H_C from BoNT/C1. This corresponds to the C-terminal domain of the H_C and has been shown to confer the ability to bind to neuronal receptors. Optionally the domain sequence is preceded at the N-terminus by the amino acids GS encoded for by an in-frame *Bam*HI site used for cloning of the fragment.

Sequence 13 In expression constructs the first alanine residue shown is preceded by a factor Xa cleavage site such that the proteolytic processing removes all of the fusion partner. The intrachain linker is simultaneously cleaved during the same processing step.

Sequence 14 shows the fusion protein C3Stau2 BoNT/C1- HC with the di-chain linker region. In expression constructs the first alanine residue shown is preceded by a factor Xa cleavage site such that the proteolytic processing removes all of the fusion partner. The intrachain linker is simultaneously cleaved during the same processing step.

Sequence 15 shows the fusion protein C3bot BoNT/C1- H_C with the linker region derived from the native BoNT/C1. In expression constructs the first alanine residue shown is preceded by a factor Xa cleavage site such that the proteolytic processing removes all of the fusion partner. The intrachain linker is simultaneously cleaved during the same processing step. This construct differs from Sequence 13 in that it does not contain the H_N domain and as such lacks a translocation function. This alters the intracellular trafficking of the C3 fragment in cells. Optionally the linker can be removed, with the C3 fragment joined directly onto the BoNT/C1-H_C fragment. The GS amino acid residues following the linker sequence are derived from the *Bam*HI site used in the construction of the fragment.

Sequences 16- 24 show a variety of alternative linkers suitable for joining the C3 entity to the BoNT/C1 HC or H_C fragment. In variant embodiments, these linkers optionally replace the native linker sequence, identified as Sequence 22. In several instances these linkers can be cleaved by a

different specific proteases allowing the sequential processing of the fusion site with Factor Xa and the intra-chain site.

CLAIMS

1. A composition, for delivery of a therapeutic agent to a neuronal cell, comprising:

the therapeutic agent, and

a neuronal cell targeting component, which component comprises a H_c domain of botulinum C_1 toxin, or a fragment, variant, or derivative thereof, which retains the function of the native H_c domain.

2. A composition according to Claim 1 further comprising a domain for translocation of the therapeutic agent into a cell.

3. A composition according to Claim 2 wherein the translocation domain is derived from a clostridial source.

4. A composition according to Claim 2 wherein the translocation domain is derived from a non-clostridial source.

5. A composition according to Claim 3 wherein the translocation domain is derived from *C. botulinum, C. butylicum, C. argentinense* or C. *tetani*.

6. A composition according to Claim 4 wherein the translocation domain is derived from diphtheria toxin, *Pseudomonas* exotoxin A, influenza virus haemagglutinin fusogenic peptides or amphiphilic peptides.

7. A composition according to Claim 2, wherein the translocation domain is derived from botulinum C_1 toxin and fragments, variants and derivatives thereof, or diphtheria toxin and fragments, variants and derivatives thereof.

8. A composition according to Claim 2 wherein the translocation domain is a membrane disrupting peptide.

9. A composition according Claim 1, wherein the therapeutic agent is

selected from the group consisting of drugs, growth factors, enzymes, DNA, modified viruses, drug release systems, or a combination thereof.

10. A composition according to Claim 9, wherein the therapeutic agent inhibits at least one member of the Rho family of GTPases.

11. A composition according to Claim 10, wherein the therapeutic agent is a C3 enzyme.

12. A composition according to Claim 11, wherein the C3 enzyme is derived from *C. botulinum, C. limosum, B. cereus, S. aureus, C. acetobutylicum, S. pyogenes, L. monocytogenes.*

13. A composition according to Claim 11 wherein the C3 enzyme is selected from the group consisting of C3Stau2, C3Stau1, and C3bot.

14. A composition according to Claim 11 wherein the C3 enzyme is selected from SEQ ID Nos: 1-10.

15. A composition according to any preceding claim wherein the H_c domain is made recombinantly.

16. A composition according any preceding claim, wherein the therapeutic agent and the H_c domain are joined to each other directly or via a linker molecule.

17. A composition according to any of Claims 2-15 wherein the therapeutic agent, the Hc domain and the translocation domain are joined to each other directly or via a linker molecule.

18. A composition according to Claim 16 or 17, wherein the linker molecule is selected from the group consisting of $(GGGGS)_2$, $(GGGGS)_3$, the interdomain linker of cellulase, PPPIEGR, collagen-like spacer, trypsin-sensitive diphtheria toxin peptide, or SEQ ID Nos: 16-24.

19. A composition according to any preceding claim wherein the

composition is a single polypeptide.

20. A composition according to any of Claims 1-18 , wherein the composition is a dichain polypeptide.

21. A composition according to any preceding claim, wherein the composition is a suspension, emulsion, solution or a freeze-dried powder.

22. A composition according to any preceding claim, wherein the construct of the invention is re-suspended or diluted in a pharmaceutically acceptable liquid.

23. A method of making a composition of the invention according to any of Claims 1-22 comprising expressing a DNA encoding the therapeutic agent and the neuronal cell targeting domain.

24. Use of the composition of any of Claims 1-22 for the manufacture of a medicament for promoting nerve regeneration.

25. A polypeptide construct comprising an inhibitor of a member of the Rho family of GTPases, for use in neuronal cell therapy.

26. A polypeptide according to Claim 25 further comprising

a neuronal cell targeting component, which component comprises a H_c domain of botulinum C_1 toxin, or a fragment, variant, or derivative thereof, which retains the function of the native H_c domain,

and a domain for translocation of the therapeutic agent into a cell.

27. A polypeptide according to Claim 26 wherein the translocation domain is derived from a clostridial source.

28. A polypeptide according to Claim 26 wherein the translocation domain is derived from a non-clostridial source.

29. A polypeptide according to Claim 27 wherein the translocation domain is derived from *C. botulinum, C. butylicum, C. argentinense* or C. *tetani*.

30. A polypeptide according to Claim 28 wherein the translocation domain is derived from diphtheria toxin, *Pseudomonas* exotoxin A, influenza virus haemagglutinin fusogenic peptides or amphiphilic peptides.

31. A polypeptide according to Claim 26, wherein the translocation domain is derived from botulinum C_1 toxin and fragments, variants and derivatives thereof, or diphtheria toxin and fragments, variants and derivatives thereof.

32. A polypeptide according to Claim 26 wherein the translocation domain is a membrane disrupting peptide.

33. A polypeptide according to Claim 25, wherein the inhibitor is a C3 enzyme.

34. A polypeptide according to Claim 33, wherein the C3 enzyme is derived from *C. botulinum, C. limosum, B. cereus, S. aureus, C. acetobutylicum, S. pyogenes, L. monocytogenes.*

35. A polypeptide according to Claim 33 wherein the C3 enzyme is selected from the group consisting of C3Stau2, C3Stau1, and C3bot.

36. A polypeptide according to Claim 33 wherein the C3 enzyme is selected from SEQ ID Nos: 1-10.

37. A polypeptide according to any of Claims 25-36 wherein the H_c domain is made recombinantly.

38. A polypeptide according any of Claims 25-37, wherein the inhibitor and the H_c domain are joined to each other directly or via a linker molecule.

39. A polypeptide according to any of Claims 26-37 wherein the inhibitor, the Hc domain and the translocation domain are joined to each other directly or via a linker molecule.

40. A polypeptide according to Claim 38 or 39, wherein the linker molecule is selected from the group consisting of $(GGGGS)_2$, $(GGGGS)_3$, the interdomain linker or cellulase, PPPIEGR, collagen-like spacer, trypsin-sensitive diphtheria toxin peptide, or SEQ ID Nos: 16-24.

41. A polypeptide according to any of Claims 25-40, wherein the polypeptide is a single chain.

42. A polypeptide according to any of Claims 25-40, wherein the polypeptide is a dichain.

43. A polypeptide according to any of Claims 25-42, wherein the polypeptide is re-suspended or diluted in a pharmaceutically acceptable liquid.

44. A polypeptide according to any of Claims 25-42, wherein the polypeptide is contained in a suspension, solution, emulsion, or a freeze-dried powder.

45. Use of a polypeptide of any of Claims 25-44, for the manufacture of a medicament for neuronal cell therapy.

46. Use of an inhibitor of a member of the Rho family of GTPases, for use in the manufacture of a medicament for neuronal cell therapy.

47. Use according to Claim 46 of a C3 enzyme.

48. Use according to Claim 47 wherein the C3 enzyme is derived from *C. botulinum, C. limosum, B. cereus, S. aureus, C. acetobutylicum, S. pyogenes, L. monocytogenes.*

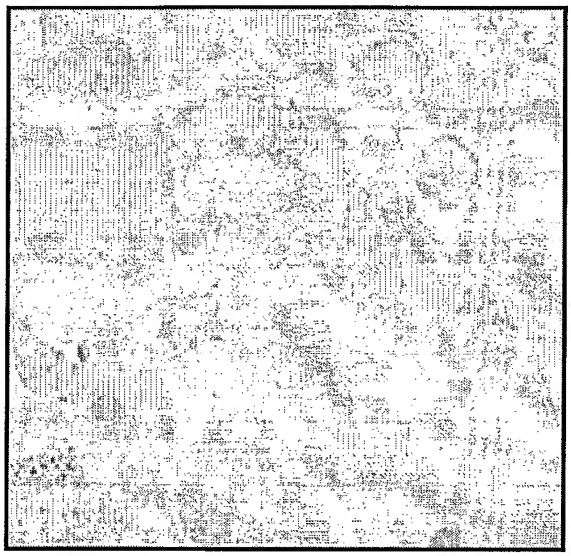
49. Use according to Claim 47 wherein the C3 enzyme is selected from the group consisting of C3Stau2, C3Stau1, and C3bot.

50. Use according to Claim 47 wherein the C3 enzyme is selected from SEQ ID Nos: 1-10.

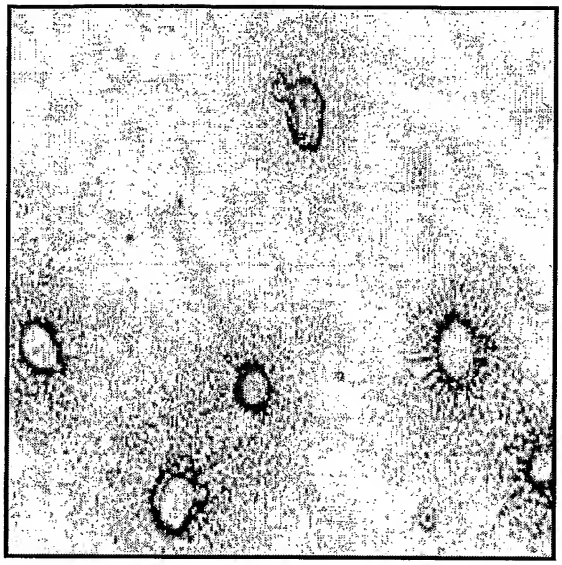
5

FIG. 1

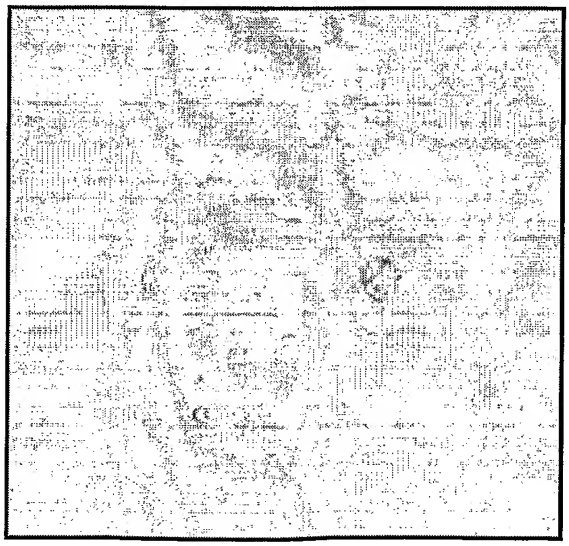
Neuroblastoma NG108 cells treated with C3 show reduced neurite retraction following subsequent treatment with LPA



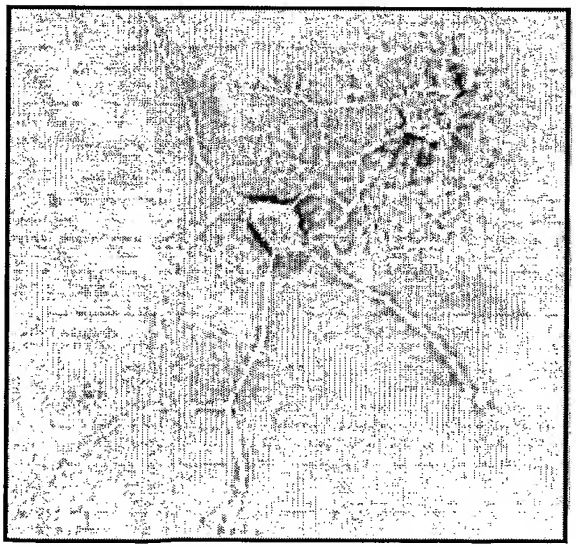
Control



1μM LPA



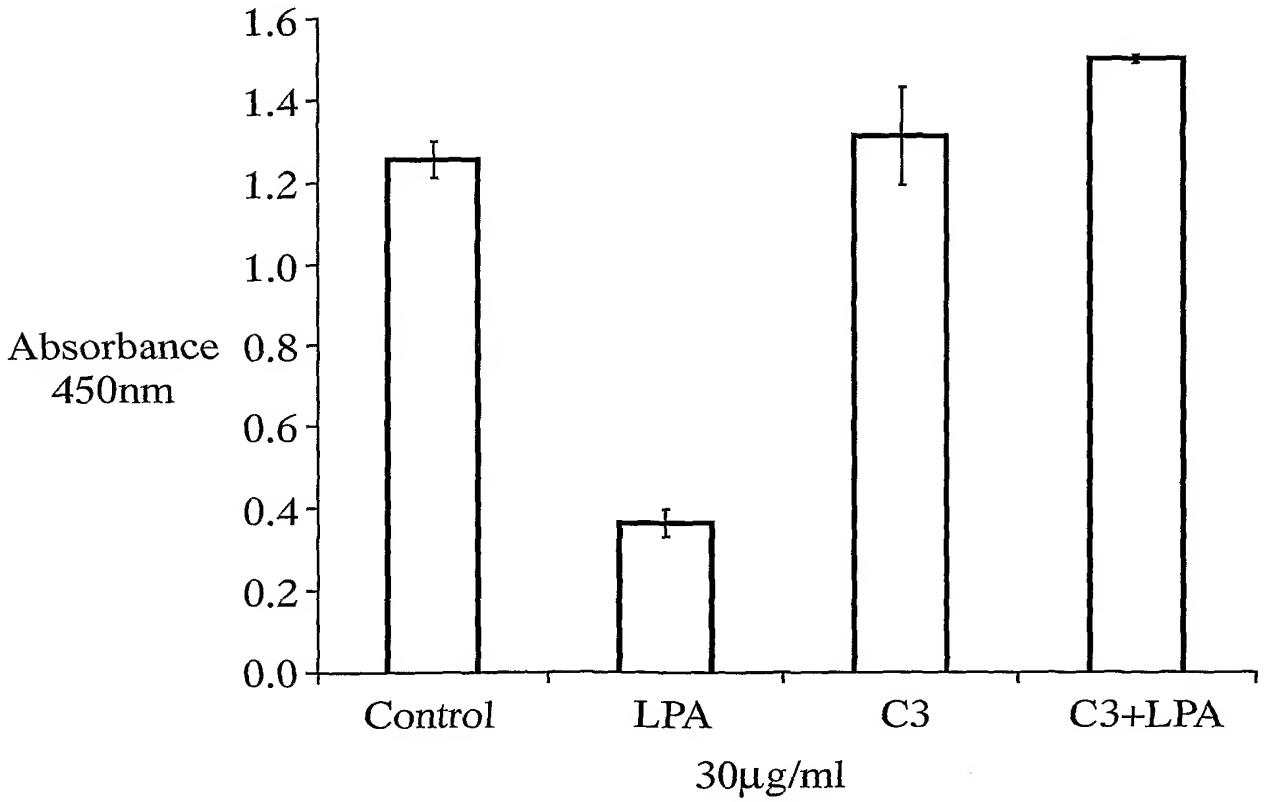
30μg/ml C3



30μg/ml C3 + 1μM LPA

FIG. 2

C3 treated cells show normal levels of mitochondrial activity following subsequent treatment with LPA



SEQUENCE LISTING

<110> Health Protection Agency

Sutton, John Shone, Clifford

<120> Targeted Agents for Nerve Regeneration

<130> GWS/CMS/24346WO

<150> GB 0216865.6

<151> 2002-07-19

<160> 24

<170> PatentIn version 3.1

<210> 1

<211> 215

<212> PRT

<213> Artificial Sequence

<220>

<223> Synthetic

<400> 1

Ile Glu Gly Arg Ala Tyr Ser Asn Thr Tyr Gln Glu Phe Thr Asn Ile
1 5 10 15

Asp Gln Ala Lys Ala Trp Gly Asn Ala Gln Tyr Lys Lys Tyr Gly Leu
20 25 30

Ser Lys Ser Glu Lys Glu Ala Ile Val Ser Tyr Thr Lys Ser Ala Ser
35 40 45

Glu Ile Asn Gly Lys Leu Arg Gln Asn Lys Gly Val Ile Asn Gly Phe
50 55 60

Pro Ser Asn Leu Ile Lys Gln Val Glu Leu Leu Asp Lys Ser Phe Asn
65 70 75 80

Lys Met Lys Thr Pro Glu Asn Ile Met Leu Phe Arg Gly Asp Asp Pro
85 90 95

Ala Tyr Leu Gly Thr Glu Phe Gln Asn Thr Leu Leu Asn Ser Asn Gly
100 105 110

Thr Ile Asn Lys Thr Ala Phe Glu Lys Ala Lys Ala Lys Phe Leu Asn
115 120 125

Lys Asp Arg Leu Glu Tyr Gly Tyr Ile Ser Thr Ser Leu Met Asn Val
130 135 140

Ser Gln Phe Ala Gly Arg Pro Ile Ile Thr Lys Phe Lys Val Ala Lys
145 150 155 160

Gly Ser Lys Ala Gly Tyr Ile Asp Pro Ile Ser Ala Phe Ala Gly Gln
165 170 175

Leu Glu Met Leu Leu Pro Arg His Ser Thr Tyr His Ile Asp Asp Met
180 185 190

Arg Leu Ser Ser Asp Gly Lys Gln Ile Ile Ile Thr Ala Thr Met Met
195 200 205

Gly Thr Ala Ile Asn Pro Lys
210 215

<210> 2

<211> 212

<212> PRT

<213> Artificial Sequence

<220>

<223> Synthetic

<400> 2

Ala Glu Thr Lys Asn Phe Thr Asp Leu Val Glu Ala Thr Lys Trp Gly
1 5 10 15

Asn Ser Leu Ile Lys Ser Ala Lys Tyr Ser Ser Lys Asp Lys Met Ala
20 25 30

Ile Tyr Asn Tyr Thr Lys Asn Ser Ser Pro Ile Asn Thr Pro Leu Arg
35 40 45

Ser Ala Asn Gly Asp Val Asn Lys Leu Ser Glu Asn Ile Gln Glu Gln
50 55 60

Val Arg Gln Leu Asp Ser Thr Ile Ser Lys Ser Val Thr Pro Asp Ser
65 70 75 80

Val Tyr Val Tyr Arg Leu Leu Asn Leu Asp Tyr Leu Ser Ser Ile Thr
85 90 95

Gly Phe Thr Arg Glu Asp Leu His Met Leu Gln Gln Thr Asn Asn Gly
100 105 110

Gln Tyr Asn Glu Ala Leu Val Ser Lys Leu Asn Asn Leu Met Asn Ser
115 120 125

Arg Ile Tyr Arg Glu Asn Gly Tyr Ser Ser Thr Gln Leu Val Ser Gly
130 135 140

Ala Ala Leu Ala Gly Arg Pro Ile Glu Leu Lys Leu Glu Leu Pro Lys
145 150 155 160

Gly Thr Lys Ala Ala Tyr Ile Asp Ser Lys Glu Leu Thr Ala Tyr Pro
165 170 175

Gly Gln Gln Glu Val Leu Leu Pro Arg Gly Thr Glu Tyr Ala Val Gly
180 185 190

Ser Val Lys Leu Ser Asp Asn Lys Arg Lys Ile Ile Ile Thr Ala Val
195 200 205

Val Phe Lys Lys
210

<210> 3

<211> 636

<212> PRT

<213> Artificial Sequence

<220>

<223> Synthetic

<400> 3

Gly Cys Thr Gly Ala Ala Ala Cys Cys Ala Ala Ala Ala Ala Cys Thr
1 5 10 15

Thr Cys Ala Cys Cys Gly Ala Cys Cys Thr Gly Gly Thr Thr Gly Ala
20 25 30

Ala Gly Cys Thr Ala Cys Cys Ala Ala Ala Thr Gly Gly Gly Gly Thr
35 40 45

Ala Ala Cys Thr Cys Thr Cys Thr Gly Ala Thr Cys Ala Ala Ala Thr
50 55 60

Cys Thr Gly Cys Thr Ala Ala Ala Thr Ala Cys Thr Cys Thr Thr Cys
65 70 75 80

Thr Ala Ala Ala Gly Ala Cys Ala Ala Ala Ala Thr Gly Gly Cys Thr
85 90 95

Ala Thr Cys Thr Ala Cys Ala Ala Cys Thr Ala Cys Ala Cys Cys Ala
100 105 110

Ala Ala Ala Ala Cys Thr Cys Thr Thr Cys Thr Cys Cys Gly Ala Thr
115 120 125

Cys Ala Ala Cys Ala Cys Cys Cys Cys Gly Cys Thr Gly Cys Gly Thr
130 135 140

```
Thr Cys Thr Gly Cys Thr Ala Ala Cys Gly Gly Thr Gly Ala Cys Gly
145                 150                 155                 160

Thr Thr Ala Ala Cys Ala Ala Ala Cys Thr Gly Thr Cys Thr Gly Ala
                165                 170                 175

Ala Ala Ala Cys Ala Thr Cys Cys Ala Gly Gly Ala Ala Cys Ala Gly
            180                 185                 190

Gly Thr Thr Cys Gly Thr Cys Ala Gly Cys Thr Gly Gly Ala Cys Thr
        195                 200                 205

Cys Thr Ala Cys Cys Ala Thr Cys Thr Cys Thr Ala Ala Ala Thr Cys
    210                 215                 220

Thr Gly Thr Thr Ala Cys Cys Cys Cys Gly Gly Ala Cys Thr Cys Thr
225                 230                 235                 240

Gly Thr Thr Thr Ala Cys Gly Thr Thr Thr Ala Cys Cys Gly Thr Cys
                245                 250                 255

Thr Gly Cys Thr Gly Ala Ala Cys Cys Thr Gly Gly Ala Cys Thr Ala
            260                 265                 270

Cys Cys Thr Gly Thr Cys Thr Thr Cys Thr Ala Thr Cys Ala Cys Cys
        275                 280                 285

Gly Gly Thr Thr Thr Cys Ala Cys Cys Cys Gly Thr Gly Ala Ala Gly
    290                 295                 300

Ala Cys Cys Thr Gly Cys Ala Cys Ala Thr Gly Cys Thr Gly Cys Ala
305                 310                 315                 320

Gly Cys Ala Gly Ala Cys Cys Ala Ala Cys Ala Ala Cys Gly Gly Thr
                325                 330                 335

Cys Ala Gly Thr Ala Cys Ala Ala Cys Gly Ala Ala Gly Cys Thr Cys
            340                 345                 350

Thr Gly Gly Thr Thr Thr Cys Thr Ala Ala Ala Cys Thr Gly Ala Ala
        355                 360                 365

Cys Ala Ala Cys Cys Thr Gly Ala Thr Gly Ala Ala Cys Thr Cys Thr
    370                 375                 380

Cys Gly Thr Ala Thr Cys Thr Ala Cys Cys Gly Thr Gly Ala Ala Ala
385                 390                 395                 400

Ala Cys Gly Gly Thr Thr Ala Cys Thr Cys Thr Thr Cys Thr Ala Cys
                405                 410                 415

Cys Cys Ala Gly Cys Thr Gly Gly Thr Thr Thr Cys Thr Gly Gly Thr
            420                 425                 430
```

Gly Cys Thr Gly Cys Thr Cys Thr Gly Gly Cys Thr Gly Gly Thr Cys
435 440 445

Gly Thr Cys Cys Gly Ala Thr Cys Gly Ala Ala Cys Thr Gly Ala Ala
450 455 460

Ala Cys Thr Gly Gly Ala Ala Cys Thr Gly Cys Cys Gly Ala Ala Ala
465 470 475 480

Gly Gly Thr Ala Cys Cys Ala Ala Ala Gly Cys Thr Gly Cys Thr Thr
485 490 495

Ala Cys Ala Thr Cys Gly Ala Cys Thr Cys Thr Ala Ala Ala Gly Ala
500 505 510

Ala Cys Thr Gly Ala Cys Cys Gly Cys Thr Thr Ala Cys Cys Cys Cys
515 520 525

Gly Gly Thr Cys Ala Gly Cys Ala Gly Gly Ala Ala Gly Thr Thr Cys
530 535 540

Thr Gly Cys Thr Gly Cys Cys Gly Cys Gly Thr Gly Gly Thr Ala Cys
545 550 555 560

Cys Gly Ala Ala Thr Ala Cys Gly Cys Thr Gly Thr Thr Gly Gly Thr
565 570 575

Thr Cys Thr Gly Thr Thr Thr Ala Ala Ala Cys Thr Gly Thr Cys Thr Gly
580 585 590

Ala Cys Ala Ala Cys Ala Ala Ala Cys Gly Thr Ala Ala Ala Ala Thr
595 600 605

Cys Ala Thr Cys Ala Thr Cys Ala Cys Cys Gly Cys Thr Gly Thr Thr
610 615 620

Gly Thr Thr Thr Thr Cys Ala Ala Gly Ala Ala Gly
625 630 635

<210> 4

<211> 212

<212> PRT

<213> Staphylococcus aureus

<400> 4

Ala Asp Val Lys Asn Phe Thr Asp Leu Asp Glu Ala Thr Lys Trp Gly
1 5 10 15

Asn Lys Leu Ile Lys Gln Ala Lys Tyr Ser Ser Asp Asp Lys Ile Ala
20 25 30

Leu Tyr Glu Tyr Thr Lys Asp Ser Ser Lys Ile Asn Gly Pro Leu Arg
35 40 45

Leu Ala Gly Gly Asp Ile Asn Lys Leu Asp Ser Thr Thr Gln Asp Lys
50 55 60

Val Arg Arg Leu Asp Ser Ser Ile Ser Lys Ser Thr Thr Pro Glu Ser
65 70 75 80

Val Tyr Val Tyr Arg Leu Leu Asn Leu Asp Tyr Leu Thr Ser Ile Val
85 90 95

Gly Phe Thr Asn Glu Asp Leu Tyr Lys Leu Gln Gln Thr Asn Asn Gly
100 105 110

Gln Tyr Asp Glu Asn Leu Val Arg Lys Leu Asn Asn Val Met Asn Ser
115 120 125

Arg Ile Tyr Arg Glu Asp Gly Tyr Ser Ser Thr Gln Leu Val Ser Gly
130 135 140

Ala Ala Val Gly Gly Arg Pro Ile Glu Leu Arg Leu Glu Leu Pro Lys
145 150 155 160

Gly Thr Lys Ala Ala Tyr Leu Asn Ser Lys Asp Leu Thr Ala Tyr Tyr
165 170 175

Gly Gln Gln Glu Val Leu Leu Pro Arg Gly Thr Glu Tyr Ala Val Gly
180 185 190

Ser Val Glu Leu Ser Asn Asp Lys Lys Lys Ile Ile Ile Thr Ala Ile
195 200 205

Val Phe Lys Lys
210

<210> 5

<211> 247

<212> PRT

<213> Staphylococcus aureus

<400> 5

Met Lys Arg Lys Leu Phe Phe Lys Ile Ile Phe Val Leu Ser Leu Val
1 5 10 15

Leu Ser Ile His Ser Ile Asn Asp Arg Thr Thr Glu Leu Ser Asn Ile
20 25 30

Ala Leu Ala Asp Asp Val Lys Asn Phe Thr Asp Leu Thr Glu Ala Thr
35 40 45

```
Asn Trp Gly Asn Lys Leu Ile Lys Gln Ala Asn Tyr Ser Ser Lys Asp
    50                  55                  60

Lys Glu Ala Ile Tyr Asn Tyr Thr Lys Tyr Ser Ser Pro Ile Asn Thr
65                  70                  75                  80

Pro Leu Arg Ser Ser Gln Gly Asp Ile Ser Asn Phe Ser Ala Asp Leu
                85                  90                  95

Gln Glu Lys Ile Leu Arg Leu Asp Arg Leu Ile Ser Lys Ser Ser Thr
            100                 105                 110

Ser Asp Ser Val Tyr Val Tyr Arg Leu Leu Asn Leu Asp Tyr Leu Ser
        115                 120                 125

Ser Val Lys Gly Phe Ser Ser Glu Asp Leu Glu Leu Leu Tyr Lys Thr
    130                 135                 140

Glu Asn Gly Lys Tyr Asn Glu Glu Leu Val Lys Lys Leu Asn Asn Ile
145                 150                 155                 160

Met Asn Ser Lys Ile Tyr Thr Glu Tyr Gly Tyr Ser Ser Thr Gln Leu
                165                 170                 175

Val Lys Gly Ala Ala Leu Ala Gly Arg Pro Ile Glu Leu Lys Leu Gln
            180                 185                 190

Leu Pro Lys Gly Thr Lys Ala Ala Tyr Ile Asp Ser Lys Asn Leu Thr
        195                 200                 205

Ala Tyr Pro Gly Gln Gln Glu Ile Leu Leu Pro Arg Gly Thr Asp Tyr
    210                 215                 220

Thr Ile Asn Thr Val Lys Leu Ser Asp Asp His Lys Arg Ile Leu Ile
225                 230                 235                 240

Glu Gly Ile Val Phe Lys Lys
                245
```

<210> 6

<211> 211

<212> PRT

<213> Clostridium limosum

<400> 6

```
Ala Tyr Ser Asn Thr Tyr Gln Glu Phe Thr Asn Ile Asp Gln Ala Lys
1               5                   10                  15

Ala Trp Gly Asn Ala Gln Tyr Lys Lys Tyr Gly Leu Ser Lys Ser Glu
            20                  25                  30
```

```
Lys Glu Ala Ile Val Ser Tyr Thr Lys Ser Ala Ser Glu Ile Asn Gly
        35                  40                  45

Lys Leu Arg Gln Asn Lys Gly Val Ile Asn Gly Phe Pro Ser Asn Leu
    50                  55                  60

Ile Lys Gln Val Glu Leu Leu Asp Lys Ser Phe Asn Lys Met Lys Thr
65                  70                  75                  80

Pro Glu Asn Ile Met Leu Phe Arg Gly Asp Asp Pro Ala Tyr Leu Gly
                85                  90                  95

Thr Glu Phe Gln Asn Thr Leu Leu Asn Ser Asn Gly Thr Ile Asn Lys
            100                 105                 110

Thr Ala Phe Glu Lys Ala Lys Ala Lys Phe Leu Asn Lys Asp Arg Leu
        115                 120                 125

Glu Tyr Gly Tyr Ile Ser Thr Ser Leu Met Asn Val Ser Gln Phe Ala
    130                 135                 140

Gly Arg Pro Ile Ile Thr Lys Phe Lys Val Ala Lys Gly Ser Lys Ala
145                 150                 155                 160

Gly Tyr Ile Asp Pro Ile Ser Ala Phe Ala Gly Gln Leu Glu Met Leu
                165                 170                 175

Leu Pro Arg His Ser Thr Tyr His Ile Asp Asp Met Arg Leu Ser Ser
            180                 185                 190

Asp Gly Lys Gln Ile Ile Ile Thr Ala Thr Met Met Gly Thr Ala Ile
        195                 200                 205

Asn Pro Lys
    210
```

<210> 7

<211> 160

<212> PRT

<213> Listeria monocytogenes

<400> 7

```
Asn Lys Ser Leu Lys Phe Thr Ser Leu Glu Glu Ser Glu Lys Trp Gly
1               5                   10                  15

Ile Asp Gly Phe Ser Val Trp Arg Asn Ser Leu Ser Ser Arg Glu Ile
            20                  25                  30

Gln Ala Ile Arg Asp Tyr Thr Asp Ile Trp His Tyr Gly Asn Met Asn
        35                  40                  45
```

Gly Tyr Leu Arg Gly Ser Val Glu Lys Leu Ala Pro Asp Asn Ala Glu
50 55 60

Arg Ile Lys Asn Leu Ser Ser Ala Leu Glu Lys Ala Glu Leu Pro Asp
65 70 75 80

Asn Ile Ile Leu Tyr Arg Gly Thr Ser Ser Glu Ile Leu Asp Asn Phe
85 90 95

Leu Asp Leu Lys Asn Leu Asn Tyr Gln Asn Leu Val Gly Lys Thr Ile
100 105 110

Glu Glu Lys Gly Phe Met Ser Thr Thr Thr Ile Ser Asn Gln Thr Phe
115 120 125

Ser Gly Asn Val Thr Met Lys Ile Asn Ala Pro Lys Gly Ser Lys Gly
130 135 140

Ala Tyr Leu Ala His Phe Ser Glu Thr Pro Glu Glu Ala Glu Val Leu
145 150 155 160

<210> 8

<211> 175

<212> PRT

<213> Clostridium:acetobutylicum

<400> 8

Thr Asn Met Asp Gln Ala Asn Glu Trp Gly Ser Gln Tyr Tyr Asp Asn
1 5 10 15

Trp Leu Lys Ser Leu Asn Asp Ser Glu Arg Asn Ala Ile Arg Gln Tyr
20 25 30

Thr Gly Asn Asp Tyr Lys Lys Ile Asn Asn Tyr Leu Arg Gly Val Asn
35 40 45

Asp Ser Leu Asp Gly Ile Asp Pro Lys Ile Ile Glu Asp Ile Lys Ser
50 55 60

Gly Leu Lys Lys Ala Ser Val Pro His Asp Met Lys Val Tyr Arg Gly
65 70 75 80

Thr Asp Leu Asn Pro Leu Arg Asn Leu Ile Asp Val Gly Lys Asp Gly
85 90 95

Ser Leu Asp Phe Ser Leu Val Gly Lys Thr Phe Lys Asp Asp Gly Phe
100 105 110

Met Ser Thr Ala Leu Val Lys Glu Ser Ser Phe Asp Tyr Met Asn Val
115 120 125

Ser Trp Glu Ile Asn Val Pro Lys Gly Thr Glu Ala Ala Tyr Val Ser
130 135 140

Lys Ile Ser Tyr Phe Pro Asp Glu Ala Glu Leu Leu Leu Asn His Gly
145 150 155 160

Gln Glu Met Ile Ile Lys Glu Ala Thr Val Gly Ser Asp Gly Lys
165 170 175

<210> 9

<211> 250

<212> PRT

<213> Streptococcus pyogenes

<400> 9

Met Leu Lys Lys Arg Tyr Gln Leu Ala Ile Val Leu Leu Leu Ser Cys
1 5 10 15

Phe Ser Leu Ile Trp Gln Thr Glu Gly Leu Val Glu Leu Phe Val Cys
20 25 30

Glu His Tyr Glu Arg Ala Val Cys Glu Gly Thr Pro Ala Tyr Phe Thr
35 40 45

Phe Ser Asp Gln Lys Gly Ala Glu Thr Leu Ile Lys Lys Arg Trp Gly
50 55 60

Lys Gly Leu Ile Tyr Pro Arg Ala Glu Gln Glu Ala Met Ala Ala Tyr
65 70 75 80

Thr Cys Gln Gln Ala Gly Pro Ile Asn Thr Ser Leu Asp Lys Ala Lys
85 90 95

Gly Glu Leu Ser Gln Leu Thr Pro Glu Leu Arg Asp Gln Val Ala Gln
100 105 110

Leu Asp Ala Ala Thr His Arg Leu Val Ile Pro Trp Asn Ile Val Val
115 120 125

Tyr Arg Tyr Val Tyr Glu Thr Phe Leu Arg Asp Ile Gly Val Ser His
130 135 140

Ala Asp Leu Thr Ser Tyr Tyr Arg Asn His Gln Phe Asp Pro His Ile
145 150 155 160

Leu Cys Lys Ile Lys Leu Gly Thr Arg Tyr Thr Lys His Ser Phe Met
165 170 175

Ser Thr Thr Ala Leu Lys Asn Gly Ala Met Thr His Arg Pro Val Glu
180 185 190

Val Arg Ile Cys Val Lys Lys Gly Ala Lys Ala Ala Phe Val Glu Pro
195 200 205

Tyr Ser Ala Val Pro Ser Glu Val Glu Leu Leu Phe Pro Arg Gly Cys
210 215 220

Gln Leu Glu Val Val Gly Ala Tyr Val Ser Gln Asp Gln Lys Lys Leu
225 230 235 240

His Ile Glu Ala Tyr Phe Lys Gly Ser Leu
245 250

<210> 10

<211> 250

<212> PRT

<213> Streptococcus pyogenes

<400> 10

Met Leu Lys Lys Arg Tyr Gln Leu Ala Ile Val Leu Leu Leu Ser Cys
1 5 10 15

Phe Ser Leu Ile Trp Gln Thr Glu Gly Leu Val Glu Leu Phe Val Cys
20 25 30

Glu His Tyr Glu Arg Ala Val Cys Glu Gly Thr Pro Ala Tyr Phe Thr
35 40 45

Phe Ser Asp Gln Lys Gly Ala Glu Thr Leu Ile Lys Lys Arg Trp Gly
50 55 60

Lys Gly Leu Ile Tyr Pro Arg Ala Glu Gln Glu Ala Met Ala Ala Tyr
65 70 75 80

Thr Cys Gln Gln Ala Gly Pro Ile Asn Thr Ser Leu Asp Lys Ala Lys
85 90 95

Gly Glu Leu Ser Gln Leu Thr Pro Glu Leu Arg Asp Gln Val Ala Gln
100 105 110

Leu Asp Ala Ala Thr His Arg Leu Val Ile Pro Trp Asn Ile Val Val
115 120 125

Tyr Arg Tyr Val Tyr Glu Thr Phe Leu Arg Asp Ile Gly Val Ser His
130 135 140

Ala Asp Leu Thr Ser Tyr Tyr Arg Asn His Gln Phe Asp Pro His Ile
145 150 155 160

Leu Cys Lys Ile Lys Leu Gly Thr Arg Tyr Thr Lys His Ser Phe Met
165 170 175

Ser Thr Thr Ala Leu Lys Asn Gly Ala Met Thr His Arg Pro Val Glu
180 185 190

Val Arg Ile Cys Val Lys Lys Gly Ala Lys Ala Ala Phe Val Glu Pro
195 200 205

Tyr Ser Ala Val Pro Ser Glu Val Glu Leu Leu Phe Pro Arg Gly Cys
210 215 220

Gln Leu Glu Val Val Gly Ala Tyr Val Ser Gln Asp Gln Lys Lys Leu
225 230 235 240

His Ile Glu Ala Tyr Phe Lys Gly Ser Leu
245 250

<210> 11

<211> 855

<212> PRT

<213> Clostridium botulinum

<400> 11

Cys His Lys Ala Ile Asp Gly Arg Ser Leu Tyr Asn Lys Thr Leu Asp
1 5 10 15

Cys Arg Glu Leu Leu Val Lys Asn Thr Asp Leu Pro Phe Ile Gly Asp
20 25 30

Ile Ser Asp Val Lys Thr Asp Ile Phe Leu Arg Lys Asp Ile Asn Glu
35 40 45

Glu Thr Glu Val Ile Tyr Tyr Pro Asp Asn Val Ser Val Asp Gln Val
50 55 60

Ile Leu Ser Lys Asn Thr Ser Glu His Gly Gln Leu Asp Leu Leu Tyr
65 70 75 80

Pro Ser Ile Asp Ser Glu Ser Glu Ile Leu Pro Gly Glu Asn Gln Val
85 90 95

Phe Tyr Asp Asn Arg Thr Gln Asn Val Asp Tyr Leu Asn Ser Tyr Tyr
100 105 110

Tyr Leu Glu Ser Gln Lys Leu Ser Asp Asn Val Glu Asp Phe Thr Phe
115 120 125

Thr Arg Ser Ile Glu Glu Ala Leu Asp Asn Ser Ala Lys Val Tyr Thr
130 135 140

Tyr Phe Pro Thr Leu Ala Asn Lys Val Asn Ala Gly Val Gln Gly Gly
145 150 155 160

Leu Phe Leu Met Trp Ala Asn Asp Val Val Glu Asp Phe Thr Thr Asn
165 170 175

Ile Leu Arg Lys Asp Thr Leu Asp Lys Ile Ser Asp Val Ser Ala Ile
180 185 190

Ile Pro Tyr Ile Gly Pro Ala Leu Asn Ile Ser Asn Ser Val Arg Arg
195 200 205

Gly Asn Phe Thr Glu Ala Phe Ala Val Thr Gly Val Thr Ile Leu Leu
210 215 220

Glu Ala Phe Pro Glu Phe Thr Ile Pro Ala Leu Gly Ala Phe Val Ile
225 230 235 240

Tyr Ser Lys Val Gln Glu Arg Asn Glu Ile Ile Lys Thr Ile Asp Asn
245 250 255

Cys Leu Glu Gln Arg Ile Lys Arg Trp Lys Asp Ser Tyr Glu Trp Met
260 265 270

Met Gly Thr Trp Leu Ser Arg Ile Ile Thr Gln Phe Asn Asn Ile Ser
275 280 285

Tyr Gln Met Tyr Asp Ser Leu Asn Tyr Gln Ala Gly Ala Ile Lys Ala
290 295 300

Lys Ile Asp Leu Glu Tyr Lys Lys Tyr Ser Gly Ser Asp Lys Glu Asn
305 310 315 320

Ile Lys Ser Gln Val Glu Asn Leu Lys Asn Ser Leu Asp Val Lys Ile
325 330 335

Ser Glu Ala Met Asn Asn Ile Asn Lys Phe Ile Arg Glu Cys Ser Val
340 345 350

Thr Tyr Leu Phe Lys Asn Met Leu Pro Lys Val Ile Asp Glu Leu Asn
355 360 365

Glu Phe Asp Arg Asn Thr Lys Ala Lys Leu Ile Asn Leu Ile Asp Ser
370 375 380

His Asn Ile Ile Leu Val Gly Glu Val Asp Lys Leu Lys Ala Lys Val
385 390 395 400

Asn Asn Ser Phe Gln Asn Thr Ile Pro Phe Asn Ile Phe Ser Tyr Thr
405 410 415

Asn Asn Ser Leu Leu Lys Asp Ile Ile Asn Glu Tyr Phe Asn Asn Ile
420 425 430

Asn Asp Ser Lys Ile Leu Ser Leu Gln Asn Arg Lys Asn Thr Leu Val
435 440 445

Asp Thr Ser Gly Tyr Asn Ala Glu Val Ser Glu Glu Gly Asp Val Gln
450 455 460

Leu Asn Pro Ile Phe Pro Phe Asp Phe Lys Leu Gly Ser Ser Gly Glu
465 470 475 480

Asp Arg Gly Lys Val Ile Val Thr Gln Asn Glu Asn Ile Val Tyr Asn
485 490 495

Ser Met Tyr Glu Ser Phe Ser Ile Ser Phe Trp Ile Arg Ile Asn Lys
500 505 510

Trp Val Ser Asn Leu Pro Gly Tyr Thr Ile Ile Asp Ser Val Lys Asn
515 520 525

Asn Ser Gly Trp Ser Ile Gly Ile Ile Ser Asn Phe Leu Val Phe Thr
530 535 540

Leu Lys Gln Asn Glu Asp Ser Glu Gln Ser Ile Asn Phe Ser Tyr Asp
545 550 555 560

Ile Ser Asn Asn Ala Pro Gly Tyr Asn Lys Trp Phe Phe Val Thr Val
565 570 575

Thr Asn Asn Met Met Gly Asn Met Lys Ile Tyr Ile Asn Gly Lys Leu
580 585 590

Ile Asp Thr Ile Lys Val Lys Glu Leu Thr Gly Ile Asn Phe Ser Lys
595 600 605

Thr Ile Thr Phe Glu Ile Asn Lys Ile Pro Asp Thr Gly Leu Ile Thr
610 615 620

Ser Asp Ser Asp Asn Ile Asn Met Trp Ile Arg Asp Phe Tyr Ile Phe
625 630 635 640

Ala Lys Glu Leu Asp Gly Lys Asp Ile Asn Ile Leu Phe Asn Ser Leu
645 650 655

Gln Tyr Thr Asn Val Val Lys Asp Tyr Trp Gly Asn Asp Leu Arg Tyr
660 665 670

Asn Lys Glu Tyr Tyr Met Val Asn Ile Asp Tyr Leu Asn Arg Tyr Met
675 680 685

Tyr Ala Asn Ser Arg Gln Ile Val Phe Asn Thr Arg Arg Asn Asn Asn
690 695 700

Asp Phe Asn Glu Gly Tyr Lys Ile Ile Ile Lys Arg Ile Arg Gly Asn
705 710 715 720

Thr Asn Asp Thr Arg Val Arg Gly Gly Asp Ile Leu Tyr Phe Asp Met
725 730 735

Thr Ile Asn Asn Lys Ala Tyr Asn Leu Phe Met Lys Asn Glu Thr Met
740 745 750

Tyr Ala Asp Asn His Ser Thr Glu Asp Ile Tyr Ala Ile Gly Leu Arg
755 760 765

Glu Gln Thr Lys Asp Ile Asn Asp Asn Ile Ile Phe Gln Ile Gln Pro
770 775 780

Met Asn Asn Thr Tyr Tyr Tyr Ala Ser Gln Ile Phe Lys Ser Asn Phe
785 790 795 800

Asn Gly Glu Asn Ile Ser Gly Ile Cys Ser Ile Gly Thr Tyr Arg Phe
805 810 815

Arg Leu Gly Gly Asp Trp Tyr Arg His Asn Tyr Leu Val Pro Thr Val
820 825 830

Lys Gln Gly Asn Tyr Ala Ser Leu Leu Glu Ser Thr Ser Thr His Trp
835 840 845

Gly Phe Val Pro Val Ser Glu
850 855

<210> 12

<211> 454

<212> PRT

<213> Clostridium botulinum

<400> 12

Gly Ser Phe Gln Asn Thr Ile Pro Phe Asn Ile Phe Ser Tyr Thr Asn
1 5 10 15

Asn Ser Leu Leu Lys Asp Ile Ile Asn Glu Tyr Phe Asn Asn Ile Asn
20 25 30

Asp Ser Lys Ile Leu Ser Leu Gln Asn Arg Lys Asn Thr Leu Val Asp
35 40 45

Thr Ser Gly Tyr Asn Ala Glu Val Ser Glu Glu Gly Asp Val Gln Leu
50 55 60

Asn Pro Ile Phe Pro Phe Asp Phe Lys Leu Gly Ser Ser Gly Glu Asp
65 70 75 80

Arg Gly Lys Val Ile Val Thr Gln Asn Glu Asn Ile Val Tyr Asn Ser
85 90 95

```
Met Tyr Glu Ser Phe Ser Ile Ser Phe Trp Ile Arg Ile Asn Lys Trp
            100                 105                 110

Val Ser Asn Leu Pro Gly Tyr Thr Ile Ile Asp Ser Val Lys Asn Asn
        115                 120                 125

Ser Gly Trp Ser Ile Gly Ile Ile Ser Asn Phe Leu Val Phe Thr Leu
    130                 135                 140

Lys Gln Asn Glu Asp Ser Glu Gln Ser Ile Asn Phe Ser Tyr Asp Ile
145                 150                 155                 160

Ser Asn Asn Ala Pro Gly Tyr Asn Lys Trp Phe Phe Val Thr Val Thr
                165                 170                 175

Asn Asn Met Met Gly Asn Met Lys Ile Tyr Ile Asn Gly Lys Leu Ile
            180                 185                 190

Asp Thr Ile Lys Val Lys Glu Leu Thr Gly Ile Asn Phe Ser Lys Thr
        195                 200                 205

Ile Thr Phe Glu Ile Asn Lys Ile Pro Asp Thr Gly Leu Ile Thr Ser
    210                 215                 220

Asp Ser Asp Asn Ile Asn Met Trp Ile Arg Asp Phe Tyr Ile Phe Ala
225                 230                 235                 240

Lys Glu Leu Asp Gly Lys Asp Ile Asn Ile Leu Phe Asn Ser Leu Gln
                245                 250                 255

Tyr Thr Asn Val Val Lys Asp Tyr Trp Gly Asn Asp Leu Arg Tyr Asn
            260                 265                 270

Lys Glu Tyr Tyr Met Val Asn Ile Asp Tyr Leu Asn Arg Tyr Met Tyr
        275                 280                 285

Ala Asn Ser Arg Gln Ile Val Phe Asn Thr Arg Arg Asn Asn Asn Asp
    290                 295                 300

Phe Asn Glu Gly Tyr Lys Ile Ile Ile Lys Arg Ile Arg Gly Asn Thr
305                 310                 315                 320

Asn Asp Thr Arg Val Arg Gly Gly Asp Ile Leu Tyr Phe Asp Met Thr
                325                 330                 335

Ile Asn Asn Lys Ala Tyr Asn Leu Phe Met Lys Asn Glu Thr Met Tyr
            340                 345                 350

Ala Asp Asn His Ser Thr Glu Asp Ile Tyr Ala Ile Gly Leu Arg Glu
        355                 360                 365

Gln Thr Lys Asp Ile Asn Asp Asn Ile Ile Phe Gln Ile Gln Pro Met
    370                 375                 380
```

Asn Asn Thr Tyr Tyr Tyr Ala Ser Gln Ile Phe Lys Ser Asn Phe Asn
385 390 395 400

Gly Glu Asn Ile Ser Gly Ile Cys Ser Ile Gly Thr Tyr Arg Phe Arg
405 410 415

Leu Gly Gly Asp Trp Tyr Arg His Asn Tyr Leu Val Pro Thr Val Lys
420 425 430

Gln Gly Asn Tyr Ala Ser Leu Leu Glu Ser Thr Ser Thr His Trp Gly
435 440 445

Phe Val Pro Val Ser Glu
450

<210> 13

<211> 1066

<212> PRT

<213> Artificial Sequence

<220>

<223> Synthetic

<400> 13

Ala Tyr Ser Asn Thr Tyr Gln Glu Phe Thr Asn Ile Asp Gln Ala Lys
1 5 10 15

Ala Trp Gly Asn Ala Gln Tyr Lys Lys Tyr Gly Leu Ser Lys Ser Glu
20 25 30

Lys Glu Ala Ile Val Ser Tyr Thr Lys Ser Ala Ser Glu Ile Asn Gly
35 40 45

Lys Leu Arg Gln Asn Lys Gly Val Ile Asn Gly Phe Pro Ser Asn Leu
50 55 60

Ile Lys Gln Val Glu Leu Leu Asp Lys Ser Phe Asn Lys Met Lys Thr
65 70 75 80

Pro Glu Asn Ile Met Leu Phe Arg Gly Asp Asp Pro Ala Tyr Leu Gly
85 90 95

Thr Glu Phe Gln Asn Thr Leu Leu Asn Ser Asn Gly Thr Ile Asn Lys
100 105 110

Thr Ala Phe Glu Lys Ala Lys Ala Lys Phe Leu Asn Lys Asp Arg Leu
115 120 125

Glu Tyr Gly Tyr Ile Ser Thr Ser Leu Met Asn Val Ser Gln Phe Ala
130 135 140

Gly Arg Pro Ile Ile Thr Lys Phe Lys Val Ala Lys Gly Ser Lys Ala
145 150 155 160

Gly Tyr Ile Asp Pro Ile Ser Ala Phe Ala Gly Gln Leu Glu Met Leu
165 170 175

Leu Pro Arg His Ser Thr Tyr His Ile Asp Asp Met Arg Leu Ser Ser
180 185 190

Asp Gly Lys Gln Ile Ile Ile Thr Ala Thr Met Met Gly Thr Ala Ile
195 200 205

Asn Pro Lys Cys His Lys Ala Ile Asp Gly Arg Ser Leu Tyr Asn Lys
210 215 220

Thr Leu Asp Cys Arg Glu Leu Leu Val Lys Asn Thr Asp Leu Pro Phe
225 230 235 240

Ile Gly Asp Ile Ser Asp Val Lys Thr Asp Ile Phe Leu Arg Lys Asp
245 250 255

Ile Asn Glu Glu Thr Glu Val Ile Tyr Tyr Pro Asp Asn Val Ser Val
260 265 270

Asp Gln Val Ile Leu Ser Lys Asn Thr Ser Glu His Gly Gln Leu Asp
275 280 285

Leu Leu Tyr Pro Ser Ile Asp Ser Glu Ser Glu Ile Leu Pro Gly Glu
290 295 300

Asn Gln Val Phe Tyr Asp Asn Arg Thr Gln Asn Val Asp Tyr Leu Asn
305 310 315 320

Ser Tyr Tyr Tyr Leu Glu Ser Gln Lys Leu Ser Asp Asn Val Glu Asp
325 330 335

Phe Thr Phe Thr Arg Ser Ile Glu Glu Ala Leu Asp Asn Ser Ala Lys
340 345 350

Val Tyr Thr Tyr Phe Pro Thr Leu Ala Asn Lys Val Asn Ala Gly Val
355 360 365

Gln Gly Gly Leu Phe Leu Met Trp Ala Asn Asp Val Val Glu Asp Phe
370 375 380

Thr Thr Asn Ile Leu Arg Lys Asp Thr Leu Asp Lys Ile Ser Asp Val
385 390 395 400

Ser Ala Ile Ile Pro Tyr Ile Gly Pro Ala Leu Asn Ile Ser Asn Ser
405 410 415

Val Arg Arg Gly Asn Phe Thr Glu Ala Phe Ala Val Thr Gly Val Thr
420 425 430

Ile Leu Leu Glu Ala Phe Pro Glu Phe Thr Ile Pro Ala Leu Gly Ala
435 440 445

Phe Val Ile Tyr Ser Lys Val Gln Glu Arg Asn Glu Ile Ile Lys Thr
450 455 460

Ile Asp Asn Cys Leu Glu Gln Arg Ile Lys Arg Trp Lys Asp Ser Tyr
465 470 475 480

Glu Trp Met Met Gly Thr Trp Leu Ser Arg Ile Ile Thr Gln Phe Asn
485 490 495

Asn Ile Ser Tyr Gln Met Tyr Asp Ser Leu Asn Tyr Gln Ala Gly Ala
500 505 510

Ile Lys Ala Lys Ile Asp Leu Glu Tyr Lys Lys Tyr Ser Gly Ser Asp
515 520 525

Lys Glu Asn Ile Lys Ser Gln Val Glu Asn Leu Lys Asn Ser Leu Asp
530 535 540

Val Lys Ile Ser Glu Ala Met Asn Asn Ile Asn Lys Phe Ile Arg Glu
545 550 555 560

Cys Ser Val Thr Tyr Leu Phe Lys Asn Met Leu Pro Lys Val Ile Asp
565 570 575

Glu Leu Asn Glu Phe Asp Arg Asn Thr Lys Ala Lys Leu Ile Asn Leu
580 585 590

Ile Asp Ser His Asn Ile Ile Leu Val Gly Glu Val Asp Lys Leu Lys
595 600 605

Ala Lys Val Asn Asn Ser Phe Gln Asn Thr Ile Pro Phe Asn Ile Phe
610 615 620

Ser Tyr Thr Asn Asn Ser Leu Leu Lys Asp Ile Ile Asn Glu Tyr Phe
625 630 635 640

Asn Asn Ile Asn Asp Ser Lys Ile Leu Ser Leu Gln Asn Arg Lys Asn
645 650 655

Thr Leu Val Asp Thr Ser Gly Tyr Asn Ala Glu Val Ser Glu Glu Gly
660 665 670

Asp Val Gln Leu Asn Pro Ile Phe Pro Phe Asp Phe Lys Leu Gly Ser
675 680 685

Ser Gly Glu Asp Arg Gly Lys Val Ile Val Thr Gln Asn Glu Asn Ile
690 695 700

Val Tyr Asn Ser Met Tyr Glu Ser Phe Ser Ile Ser Phe Trp Ile Arg
705 710 715 720

Ile Asn Lys Trp Val Ser Asn Leu Pro Gly Tyr Thr Ile Ile Asp Ser
725 730 735

Val Lys Asn Asn Ser Gly Trp Ser Ile Gly Ile Ile Ser Asn Phe Leu
740 745 750

Val Phe Thr Leu Lys Gln Asn Glu Asp Ser Glu Gln Ser Ile Asn Phe
755 760 765

Ser Tyr Asp Ile Ser Asn Asn Ala Pro Gly Tyr Asn Lys Trp Phe Phe
770 775 780

Val Thr Val Thr Asn Asn Met Met Gly Asn Met Lys Ile Tyr Ile Asn
785 790 795 800

Gly Lys Leu Ile Asp Thr Ile Lys Val Lys Glu Leu Thr Gly Ile Asn
805 810 815

Phe Ser Lys Thr Ile Thr Phe Glu Ile Asn Lys Ile Pro Asp Thr Gly
820 825 830

Leu Ile Thr Ser Asp Ser Asp Asn Ile Asn Met Trp Ile Arg Asp Phe
835 840 845

Tyr Ile Phe Ala Lys Glu Leu Asp Gly Lys Asp Ile Asn Ile Leu Phe
850 855 860

Asn Ser Leu Gln Tyr Thr Asn Val Val Lys Asp Tyr Trp Gly Asn Asp
865 870 875 880

Leu Arg Tyr Asn Lys Glu Tyr Tyr Met Val Asn Ile Asp Tyr Leu Asn
885 890 895

Arg Tyr Met Tyr Ala Asn Ser Arg Gln Ile Val Phe Asn Thr Arg Arg
900 905 910

Asn Asn Asn Asp Phe Asn Glu Gly Tyr Lys Ile Ile Ile Lys Arg Ile
915 920 925

Arg Gly Asn Thr Asn Asp Thr Arg Val Arg Gly Gly Asp Ile Leu Tyr
930 935 940

Phe Asp Met Thr Ile Asn Asn Lys Ala Tyr Asn Leu Phe Met Lys Asn
945 950 955 960

Glu Thr Met Tyr Ala Asp Asn His Ser Thr Glu Asp Ile Tyr Ala Ile
965 970 975

Gly Leu Arg Glu Gln Thr Lys Asp Ile Asn Asp Asn Ile Ile Phe Gln
980 985 990

Ile Gln Pro Met Asn Asn Thr Tyr Tyr Tyr Ala Ser Gln Ile Phe Lys
995 1000 1005

Ser Asn Phe Asn Gly Glu Asn Ile Ser Gly Ile Cys Ser Ile Gly
1010 1015 1020

Thr Tyr Arg Phe Arg Leu Gly Gly Asp Trp Tyr Arg His Asn Tyr
1025 1030 1035

Leu Val Pro Thr Val Lys Gln Gly Asn Tyr Ala Ser Leu Leu Glu
1040 1045 1050

Ser Thr Ser Thr His Trp Gly Phe Val Pro Val Ser Glu
1055 1060 1065

<210> 14

<211> 1067

<212> PRT

<213> Artificial Sequence

<220>

<223> Synthetic

<400> 14

Ala Glu Thr Lys Asn Phe Thr Asp Leu Val Glu Ala Thr Lys Trp Gly
1 5 10 15

Asn Ser Leu Ile Lys Ser Ala Lys Tyr Ser Ser Lys Asp Lys Met Ala
20 25 30

Ile Tyr Asn Tyr Thr Lys Asn Ser Ser Pro Ile Asn Thr Pro Leu Arg
35 40 45

Ser Ala Asn Gly Asp Val Asn Lys Leu Ser Glu Asn Ile Gln Glu Gln
50 55 60

Val Arg Gln Leu Asp Ser Thr Ile Ser Lys Ser Val Thr Pro Asp Ser
65 70 75 80

Val Tyr Val Tyr Arg Leu Leu Asn Leu Asp Tyr Leu Ser Ser Ile Thr
85 90 95

Gly Phe Thr Arg Glu Asp Leu His Met Leu Gln Gln Thr Asn Asn Gly
100 105 110

Gln Tyr Asn Glu Ala Leu Val Ser Lys Leu Asn Asn Leu Met Asn Ser
115 120 125

Arg Ile Tyr Arg Glu Asn Gly Tyr Ser Ser Thr Gln Leu Val Ser Gly
130 135 140

Ala Ala Leu Ala Gly Arg Pro Ile Glu Leu Lys Leu Glu Leu Pro Lys
145 150 155 160

Gly Thr Lys Ala Ala Tyr Ile Asp Ser Lys Glu Leu Thr Ala Tyr Pro
165 170 175

Gly Gln Gln Glu Val Leu Leu Pro Arg Gly Thr Glu Tyr Ala Val Gly
180 185 190

Ser Val Lys Leu Ser Asp Asn Lys Arg Lys Ile Ile Ile Thr Ala Val
195 200 205

Val Phe Lys Lys Cys His Lys Ala Ile Asp Gly Arg Ser Leu Tyr Asn
210 215 220

Lys Thr Leu Asp Cys Arg Glu Leu Leu Val Lys Asn Thr Asp Leu Pro
225 230 235 240

Phe Ile Gly Asp Ile Ser Asp Val Lys Thr Asp Ile Phe Leu Arg Lys
245 250 255

Asp Ile Asn Glu Glu Thr Glu Val Ile Tyr Tyr Pro Asp Asn Val Ser
260 265 270

Val Asp Gln Val Ile Leu Ser Lys Asn Thr Ser Glu His Gly Gln Leu
275 280 285

Asp Leu Leu Tyr Pro Ser Ile Asp Ser Glu Ser Glu Ile Leu Pro Gly
290 295 300

Glu Asn Gln Val Phe Tyr Asp Asn Arg Thr Gln Asn Val Asp Tyr Leu
305 310 315 320

Asn Ser Tyr Tyr Tyr Leu Glu Ser Gln Lys Leu Ser Asp Asn Val Glu
325 330 335

Asp Phe Thr Phe Thr Arg Ser Ile Glu Glu Ala Leu Asp Asn Ser Ala
340 345 350

Lys Val Tyr Thr Tyr Phe Pro Thr Leu Ala Asn Lys Val Asn Ala Gly
355 360 365

Val Gln Gly Gly Leu Phe Leu Met Trp Ala Asn Asp Val Val Glu Asp
370 375 380

Phe Thr Thr Asn Ile Leu Arg Lys Asp Thr Leu Asp Lys Ile Ser Asp
385 390 395 400

Val Ser Ala Ile Ile Pro Tyr Ile Gly Pro Ala Leu Asn Ile Ser Asn
405 410 415

Ser Val Arg Arg Gly Asn Phe Thr Glu Ala Phe Ala Val Thr Gly Val
420 425 430

Thr Ile Leu Leu Glu Ala Phe Pro Glu Phe Thr Ile Pro Ala Leu Gly
435 440 445

Ala Phe Val Ile Tyr Ser Lys Val Gln Glu Arg Asn Glu Ile Ile Lys
450 455 460

Thr Ile Asp Asn Cys Leu Glu Gln Arg Ile Lys Arg Trp Lys Asp Ser
465 470 475 480

Tyr Glu Trp Met Met Gly Thr Trp Leu Ser Arg Ile Ile Thr Gln Phe
485 490 495

Asn Asn Ile Ser Tyr Gln Met Tyr Asp Ser Leu Asn Tyr Gln Ala Gly
500 505 510

Ala Ile Lys Ala Lys Ile Asp Leu Glu Tyr Lys Lys Tyr Ser Gly Ser
515 520 525

Asp Lys Glu Asn Ile Lys Ser Gln Val Glu Asn Leu Lys Asn Ser Leu
530 535 540

Asp Val Lys Ile Ser Glu Ala Met Asn Asn Ile Asn Lys Phe Ile Arg
545 550 555 560

Glu Cys Ser Val Thr Tyr Leu Phe Lys Asn Met Leu Pro Lys Val Ile
565 570 575

Asp Glu Leu Asn Glu Phe Asp Arg Asn Thr Lys Ala Lys Leu Ile Asn
580 585 590

Leu Ile Asp Ser His Asn Ile Ile Leu Val Gly Glu Val Asp Lys Leu
595 600 605

Lys Ala Lys Val Asn Asn Ser Phe Gln Asn Thr Ile Pro Phe Asn Ile
610 615 620

Phe Ser Tyr Thr Asn Asn Ser Leu Leu Lys Asp Ile Ile Asn Glu Tyr
625 630 635 640

Phe Asn Asn Ile Asn Asp Ser Lys Ile Leu Ser Leu Gln Asn Arg Lys
645 650 655

Asn Thr Leu Val Asp Thr Ser Gly Tyr Asn Ala Glu Val Ser Glu Glu
660 665 670

Gly Asp Val Gln Leu Asn Pro Ile Phe Pro Phe Asp Phe Lys Leu Gly
675 680 685

Ser Ser Gly Glu Asp Arg Gly Lys Val Ile Val Thr Gln Asn Glu Asn
690 695 700

Ile Val Tyr Asn Ser Met Tyr Glu Ser Phe Ser Ile Ser Phe Trp Ile
705 710 715 720

Arg Ile Asn Lys Trp Val Ser Asn Leu Pro Gly Tyr Thr Ile Ile Asp
725 730 735

Ser Val Lys Asn Asn Ser Gly Trp Ser Ile Gly Ile Ile Ser Asn Phe
740 745 750

Leu Val Phe Thr Leu Lys Gln Asn Glu Asp Ser Glu Gln Ser Ile Asn
755 760 765

Phe Ser Tyr Asp Ile Ser Asn Asn Ala Pro Gly Tyr Asn Lys Trp Phe
770 775 780

Phe Val Thr Val Thr Asn Asn Met Met Gly Asn Met Lys Ile Tyr Ile
785 790 795 800

Asn Gly Lys Leu Ile Asp Thr Ile Lys Val Lys Glu Leu Thr Gly Ile
805 810 815

Asn Phe Ser Lys Thr Ile Thr Phe Glu Ile Asn Lys Ile Pro Asp Thr
820 825 830

Gly Leu Ile Thr Ser Asp Ser Asp Asn Ile Asn Met Trp Ile Arg Asp
835 840 845

Phe Tyr Ile Phe Ala Lys Glu Leu Asp Gly Lys Asp Ile Asn Ile Leu
850 855 860

Phe Asn Ser Leu Gln Tyr Thr Asn Val Val Lys Asp Tyr Trp Gly Asn
865 870 875 880

Asp Leu Arg Tyr Asn Lys Glu Tyr Tyr Met Val Asn Ile Asp Tyr Leu
885 890 895

Asn Arg Tyr Met Tyr Ala Asn Ser Arg Gln Ile Val Phe Asn Thr Arg
900 905 910

Arg Asn Asn Asn Asp Phe Asn Glu Gly Tyr Lys Ile Ile Ile Lys Arg
915 920 925

Ile Arg Gly Asn Thr Asn Asp Thr Arg Val Arg Gly Gly Asp Ile Leu
930 935 940

Tyr Phe Asp Met Thr Ile Asn Asn Lys Ala Tyr Asn Leu Phe Met Lys
945 950 955 960

Asn Glu Thr Met Tyr Ala Asp Asn His Ser Thr Glu Asp Ile Tyr Ala
965 970 975

Ile Gly Leu Arg Glu Gln Thr Lys Asp Ile Asn Asp Asn Ile Ile Phe
980 985 990

Gln Ile Gln Pro Met Asn Asn Thr Tyr Tyr Tyr Ala Ser Gln Ile Phe
995 1000 1005

Lys Ser Asn Phe Asn Gly Glu Asn Ile Ser Gly Ile Cys Ser Ile
1010 1015 1020

Gly Thr Tyr Arg Phe Arg Leu Gly Gly Asp Trp Tyr Arg His Asn
1025 1030 1035

Tyr Leu Val Pro Thr Val Lys Gln Gly Asn Tyr Ala Ser Leu Leu
1040 1045 1050

Glu Ser Thr Ser Thr His Trp Gly Phe Val Pro Val Ser Glu
1055 1060 1065

<210> 15

<211> 682

<212> PRT

<213> Artificial Sequence

<220>

<223> Synthetic

<400> 15

Ala Tyr Ser Asn Thr Tyr Gln Glu Phe Thr Asn Ile Asp Gln Ala Lys
1 5 10 15

Ala Trp Gly Asn Ala Gln Tyr Lys Lys Tyr Gly Leu Ser Lys Ser Glu
20 25 30

Lys Glu Ala Ile Val Ser Tyr Thr Lys Ser Ala Ser Glu Ile Asn Gly
35 40 45

Lys Leu Arg Gln Asn Lys Gly Val Ile Asn Gly Phe Pro Ser Asn Leu
50 55 60

Ile Lys Gln Val Glu Leu Leu Asp Lys Ser Phe Asn Lys Met Lys Thr
65 70 75 80

Pro Glu Asn Ile Met Leu Phe Arg Gly Asp Asp Pro Ala Tyr Leu Gly
85 90 95

Thr Glu Phe Gln Asn Thr Leu Leu Asn Ser Asn Gly Thr Ile Asn Lys
100 105 110

Thr Ala Phe Glu Lys Ala Lys Ala Lys Phe Leu Asn Lys Asp Arg Leu
115 120 125

Glu Tyr Gly Tyr Ile Ser Thr Ser Leu Met Asn Val Ser Gln Phe Ala
130 135 140

Gly Arg Pro Ile Ile Thr Lys Phe Lys Val Ala Lys Gly Ser Lys Ala
145 150 155 160

Gly Tyr Ile Asp Pro Ile Ser Ala Phe Ala Gly Gln Leu Glu Met Leu
165 170 175

Leu Pro Arg His Ser Thr Tyr His Ile Asp Asp Met Arg Leu Ser Ser
180 185 190

Asp Gly Lys Gln Ile Ile Ile Thr Ala Thr Met Met Gly Thr Ala Ile
195 200 205

Asn Pro Lys Cys His Lys Ala Ile Asp Gly Arg Ser Leu Tyr Asn Lys
210 215 220

Thr Leu Asp Cys Gly Ser Phe Gln Asn Thr Ile Pro Phe Asn Ile Phe
225 230 235 240

Ser Tyr Thr Asn Asn Ser Leu Leu Lys Asp Ile Ile Asn Glu Tyr Phe
245 250 255

Asn Asn Ile Asn Asp Ser Lys Ile Leu Ser Leu Gln Asn Arg Lys Asn
260 265 270

Thr Leu Val Asp Thr Ser Gly Tyr Asn Ala Glu Val Ser Glu Glu Gly
275 280 285

Asp Val Gln Leu Asn Pro Ile Phe Pro Phe Asp Phe Lys Leu Gly Ser
290 295 300

Ser Gly Glu Asp Arg Gly Lys Val Ile Val Thr Gln Asn Glu Asn Ile
305 310 315 320

Val Tyr Asn Ser Met Tyr Glu Ser Phe Ser Ile Ser Phe Trp Ile Arg
325 330 335

Ile Asn Lys Trp Val Ser Asn Leu Pro Gly Tyr Thr Ile Ile Asp Ser
340 345 350

Val Lys Asn Asn Ser Gly Trp Ser Ile Gly Ile Ile Ser Asn Phe Leu
355 360 365

Val Phe Thr Leu Lys Gln Asn Glu Asp Ser Glu Gln Ser Ile Asn Phe
370 375 380

Ser Tyr Asp Ile Ser Asn Asn Ala Pro Gly Tyr Asn Lys Trp Phe Phe
385 390 395 400

Val Thr Val Thr Asn Asn Met Met Gly Asn Met Lys Ile Tyr Ile Asn
405 410 415

Gly Lys Leu Ile Asp Thr Ile Lys Val Lys Glu Leu Thr Gly Ile Asn
420 425 430

Phe Ser Lys Thr Ile Thr Phe Glu Ile Asn Lys Ile Pro Asp Thr Gly
435 440 445

Leu Ile Thr Ser Asp Ser Asp Asn Ile Asn Met Trp Ile Arg Asp Phe
450 455 460

Tyr Ile Phe Ala Lys Glu Leu Asp Gly Lys Asp Ile Asn Ile Leu Phe
465 470 475 480

Asn Ser Leu Gln Tyr Thr Asn Val Val Lys Asp Tyr Trp Gly Asn Asp
485 490 495

Leu Arg Tyr Asn Lys Glu Tyr Tyr Met Val Asn Ile Asp Tyr Leu Asn
500 505 510

Arg Tyr Met Tyr Ala Asn Ser Arg Gln Ile Val Phe Asn Thr Arg Arg
515 520 525

Asn Asn Asn Asp Phe Asn Glu Gly Tyr Lys Ile Ile Ile Lys Arg Ile
530 535 540

Arg Gly Asn Thr Asn Asp Thr Arg Val Arg Gly Gly Asp Ile Leu Tyr
545 550 555 560

Phe Asp Met Thr Ile Asn Asn Lys Ala Tyr Asn Leu Phe Met Lys Asn
565 570 575

Glu Thr Met Tyr Ala Asp Asn His Ser Thr Glu Asp Ile Tyr Ala Ile
580 585 590

Gly Leu Arg Glu Gln Thr Lys Asp Ile Asn Asp Asn Ile Ile Phe Gln
595 600 605

Ile Gln Pro Met Asn Asn Thr Tyr Tyr Tyr Ala Ser Gln Ile Phe Lys
610 615 620

Ser Asn Phe Asn Gly Glu Asn Ile Ser Gly Ile Cys Ser Ile Gly Thr
625 630 635 640

Tyr Arg Phe Arg Leu Gly Gly Asp Trp Tyr Arg His Asn Tyr Leu Val
645 650 655

Pro Thr Val Lys Gln Gly Asn Tyr Ala Ser Leu Leu Glu Ser Thr Ser
660 665 670

Thr His Trp Gly Phe Val Pro Val Ser Glu
675 680

<210> 16

<211> 12

<212> PRT

<213> Artificial Sequence

<220>

<223> Synthetic

<400> 16

Cys Ser Ala Ile Glu Gly Arg Ala Pro Gly Ile Cys
1 5 10

<210> 17

<211> 20

<212> PRT

<213> Artificial Sequence

<220>

<223> Synthetic

<400> 17

Cys Gly Ile Glu Gly Arg Ala Pro Gly Pro Gly Ser Ser Val Gly Ser
1 5 10 15

Ser Leu Ser Cys
20

<210> 18

<211> 11

<212> PRT

<213> Artificial Sequence

<220>

<223> Synthetic

<400> 18

Cys Gly Leu Val Pro Arg Gly Ser Gly Pro Cys
1 5 10

<210> 19

<211> 20

<212> PRT

<213> Artificial Sequence

<220>

<223> Synthetic

<400> 19

Cys Gly Leu Val Pro Arg Gly Ser Gly Pro Gly Ser Ser Val Gly Ser
1 5 10 15

Ser Leu Ser Cys
20

<210> 20

<211> 13

<212> PRT

<213> Artificial Sequence

<220>

<223> Synthetic

<400> 20

Cys Lys Ser Asp Asp Asp Asp Lys Ala Pro Gly Ile Cys
1 5 10

<210> 21

<211> 22

<212> PRT

<213> Artificial Sequence

<220>

<223> Synthetic

<400> 21

Cys Lys Ser Glu Glu Lys Leu Tyr Asp Asp Asp Asp Lys Asp Arg Trp
1 5 10 15

Gly Ser Ser Arg Ile Cys
20

<210> 22

<211> 17

<212> PRT

<213> Clostridium Botulinum

<400> 22

Cys His Lys Ala Ile Asp Gly Arg Ser Leu Tyr Asn Lys Thr Leu Asp
1 5 10 15

Cys

<210> 23

<211> 10

<212> PRT

<213> Artificial Sequence

<220>

<223> Synthetic

<400> 23

Cys Gly Leu Val Pro Ala Gly Ser Gly Pro
1 5 10

<210> 24

<211> 17

<212> PRT

<213> Artificial Sequence

<220>

<223> Synthetic

<400> 24

Cys Gly Leu Val Pro Ala Gly Ser Gly Pro Ser Ala Gly Ser Ser Ala
1 5 10 15

Cys

INTERNATIONAL SEARCH REPORT

Internatic pplication No
PCT/GB 03/03082

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K47/48 C07K19/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, BIOSIS, PAJ, EMBASE, SEQUENCE SEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 00 28041 A (MICROBIOLOGICAL RES AUTHORITY ;HALLIS BASSAM (GB); SILMAN NIGEL (G) 18 May 2000 (2000-05-18)	1-9, 15-24
Y	abstract page 3, line 26,27 page 4, line 3 -page 6, line 2 page 6, line 15-21 page 7, line 15-21 page 8, line 5-13 page 9, line 6-11 page 10, line 7 -page 11, line 17 page 12, line 33 -page 14, line 23 example 3 claims 1-22	10-14

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 December 2003	05/01/2004

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Chavanne, F

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with Indication,where appropriate, of the relevant passages	Relevant to claim No.
X	WO 01 36588 A (BROWN ROBERT H JR ;GEN HOSPITAL CORP (US); MURPHY JOHN R (US); UNI) 25 May 2001 (2001-05-25)	1-9, 15-24
Y	abstract page 2, line 20-22 page 3, line 8-17 page 4, line 18-22 page 8, line 13-22 page 9, line 6-22 page 19, line 15 -page 20, line 8 claims 1-15 figure 4A	10-14

X	WO 01 58936 A (MICROBIOLOGICAL RES AUTHORITY ;SILMAN NIGEL (GB); SHONE CLIFFORD C) 16 August 2001 (2001-08-16)	1-6,8,9, 15-24
Y	abstract page 1, line 5-7 page 4, line 27 -page 5, line 24 page 6, line 8-22 page 7, line 5-18 page 7, line 31 -page 8, line 16 page 10, line 9 -page 12, line 24 figures 3-5 examples 1-6,9 claims 1-31	10-14

Y	WO 94 21300 A (FOSTER KEITH ALAN ;NORTH JOHN ROBERT (GB); HEALTH LAB SERVICE BOAR) 29 September 1994 (1994-09-29) abstract page 5, paragraph 5 page 8, paragraphs 3-5 page 9, paragraph 1 page 10, paragraphs 1-5 examples 4,6	1-24

Y	US 5 965 406 A (MURPHY JOHN R) 12 October 1999 (1999-10-12) abstract figures 1,3 column 2, line 30 -column 3, line 8 column 3, line 37-48 example 1	1-24

	-/--	

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	CHADDOCK J A ET AL: "INHIBITION OF VESICULAR SECRETION IN BOTH NEURONAL AND NONNEURONAL CELLS BY A RETARGETED ENDOPEPTIDASE DERIVATIVE OF CLOSTRIDIUM BOTULINUM NEUROTOXIN TYPE A" , INFECTION AND IMMUNITY, AMERICAN SOCIETY FOR MICROBIOLOGY. WASHINGTON, US, VOL. 68, NR. 5, PAGE(S) 2587-2593 XP001010268 ISSN: 0019-9567 abstract page 2587, column 1, paragraph 2 page 2587, column 2, paragraph 1 page 2592, column 1, paragraph 1 page 2592, column 2 ---	1-9, 15-24
X	WO 99 08533 A (UNIV YALE ;STRITTMATTER STEPHEN M (US)) 25 February 1999 (1999-02-25)	25-50
Y	abstract page 5, line 5-22 page 10, line 2 -page 11, line 12 examples 1,2 claims 1-20 -----	10-14

INTERNATIONAL SEARCH REPORT

Internati pplication No
PCT/GB 03/03082

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 0028041 A	18-05-2000	AU 754883 B2	28-11-2002
		AU 1059400 A	29-05-2000
		CA 2349496 A1	18-05-2000
		EP 1127136 A1	29-08-2001
		WO 0028041 A1	18-05-2000
		JP 2002529093 T	10-09-2002
WO 0136588 A	25-05-2001	CA 2392208 A1	25-05-2001
		WO 0136588 A2	25-05-2001
WO 0158936 A	16-08-2001	AU 1719301 A	20-08-2001
		CA 2392202 A1	16-08-2001
		EP 1234043 A2	28-08-2002
		WO 0158936 A2	16-08-2001
		JP 2003522199 T	22-07-2003
		US 2003147895 A1	07-08-2003
WO 9421300 A	29-09-1994	AT 228856 T	15-12-2002
		AU 671203 B2	15-08-1996
		AU 6217594 A	11-10-1994
		BR 9406232 A	09-01-1996
		CA 2158647 A1	29-09-1994
		CN 1124929 A	19-06-1996
		CZ 9502426 A3	17-04-1996
		DE 69431832 D1	16-01-2003
		DE 69431832 T2	10-04-2003
		DK 689459 T3	24-03-2003
		EP 0689459 A1	03-01-1996
		ES 2183836 T3	01-04-2003
		FI 954390 A	17-11-1995
		WO 9421300 A2	29-09-1994
		HU 76556 A2	29-09-1997
		IN 178499 A1	03-05-1997
		JP 9500867 T	28-01-1997
		NO 953643 A	16-11-1995
		NZ 262484 A	27-08-1996
		PL 310698 A1	27-12-1995
		PT 689459 T	30-04-2003
		SG 73411 A1	20-06-2000
		SK 115595 A3	05-02-1997
US 5965406 A	12-10-1999	US 5668255 A	16-09-1997
		US 6022950 A	08-02-2000
		AU 657087 B2	02-03-1995
		AU 7168991 A	24-07-1991
		AU 8032194 A	27-04-1995
		CA 2071969 A1	23-06-1991
		EP 0439954 A2	07-08-1991
		JP 5502880 T	20-05-1993
		NO 922447 A	19-08-1992
		WO 9109871 A1	11-07-1991
		AT 79136 T	15-08-1992
		AU 592310 B2	11-01-1990
		AU 4491685 A	10-01-1986
		CA 1339359 C	26-08-1997
		DE 3586456 D1	10-09-1992
		DE 3586456 T2	25-03-1993
		EP 0185076 A1	25-06-1986

INTERNATIONAL SEARCH REPORT

Internati pplication No
PCT/GB 03/03082

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5965406 A		ES 8708014 A1	16-11-1987
		ES 8801376 A1	01-03-1988
		FI 860541 A	06-02-1986
		JP 6205684 A	26-07-1994
		JP 2510984 B2	26-06-1996
		JP 61502304 T	16-10-1986
		NO 860421 A ,B,	06-02-1986
		NZ 212312 A	29-09-1988
		WO 8600090 A1	03-01-1986
WO 9908533 A	25-02-1999	AU 735607 B2	12-07-2001
		AU 9104298 A	08-03-1999
		CA 2300878 A1	25-02-1999
		EP 1011330 A1	28-06-2000
		JP 2001515018 T	18-09-2001
		WO 9908533 A1	25-02-1999